Cellular Signalling Vol. 1, No. 4, pp. 387–393, 1989.
Printed in Great Britain.

0898–6568/89 $3.00 + .00

STAPHYLOCOCCUS AUREUS α-TOXIN ACTIVATES PHOSPHOLIPASES AND INDUCES A Ca^{2+} INFLUX IN PC12 CELLS

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(*Received* 10 *August* 1988; *and accepted* 11 *January* 1989)

Abstract—Staphylococcal α-toxin at subcytotoxic concentrations stimulated phosphatidylinositol turnover and arachidonic acid release in undifferentiated cultures of pheochromocytoma PC12 cells. Stimulation of phospholipase A_2 but not C was dependent on extracellular calcium. Addition of staphylococcal α-toxin to PC12 cells caused a dose-dependent, biphasic increase in intracellular calcium measured by fura-2 fluorescence technique. Elevation of intracellular Ca^{2+} content occurred with a time course similar to those observed for stimulation of phospholipase A_2. Alteration of membrane structure and formation of staphylococcal α-toxin pores facilitating an influx of Ca^{2+}, represent the probable mechanisms by which phospholipases C and A_2 are activated, respectively. These results suggest a possible involvement of Ca^{2+}, phosphoinositides and arachidonic acid metabolites in the pathogenic action of staphylococcus α-toxin and caution against the general usage of this toxin as a permeabilizing agent to study stimulus-secretion coupling in secretory cells.

Key words: Staphylococcal α-toxin, intracellular Ca^{2+}, PI turnover, arachidonic acid release, cytotoxicity.

INTRODUCTION

Staphylococcus aureus α-toxin is a 33,000 dalton mol.wt exotoxin secreted by pathogenic staphylococci [1]. This toxin possesses dermanecrotic activity and is lethal in animals, probably due to interaction and perturbation of the cell membrane organization resulting in cytolysis [2]. Following binding of α-toxin to artificial or biological membranes a self-oligomerization process occurs that creates ring structures thought to represent transmembrane pores that are permeable to ions and small metabolites [3, 4]. The property of pore formation and subsequent ion permeability has been utilized in recent studies to determine molecular requirements for stimulus-secretion coupling in secretory cells permeabilized with staphylococcal α-toxin [5, 6]. The pronounced lethal effect of α-toxin has been related to both circulatory perturbations and neurotoxicity [2]. Neurotoxic effects of α-toxin, expressed by abnormal brain bioelectric activity [7], have been attributed to toxin binding and disruption of both peripheral and central nerve myelin sheaths [8]. Presently, staphylococcal α-toxin-induced neurotoxicity is believed to be the result of increased myelin protein phosphorylation [9]. Molecular studies on the pathogenic action of α-toxin in normal and/or permeabilized neuronal cells have not been reported. In this study we present evidence suggesting that staphylococcus α-toxin at subcytotoxic, permeabilizing concentrations, stimulates cellular phospholipases and induces a Ca^{2+} influx, signals which could mediate initial pathogenic events of this toxin.

Abbreviations: PC12, pheochromocytoma cell cultures; DMEM, Dulbecco's modified Eagles medium; PBS, phosphate buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; staph-α, staphylococcal α-toxin; AA, arachidonic acid; IP, phosphoinositide; $[Ca^{2+}]_{in}$, concentration of free Ca^{2+} in the cytosol; PhA_2, phospholipase A_2; PhC, phospholipase C.

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MATERIALS AND METHODS

Toxin and materials

Staphylococcus aureus α-toxin was prepared to a high degree of homogeneity as previously described [10] and had a specific activity of 30,000 hemolytic U/mg protein [10]. Carbachol was purchased from Sigma. $Na_2{}^{51}CrO_4$ was purchased from Amersham, [^{3}H] inositol from ARC (American Radiolabelled Chemicals), [^{3}H]-arachidonic acid from NEN (New England Nuclear) and Fura-2 from molecular probes.

Cell cultures

PC12 cells were cultured at 37°C as monolayers in 150 cm^2 flasks in Dulbecco's modified Eagle's medium (Gibco Lab., Grand Island, N.Y.) supplemented with 7% fetal bovine serum, 7% horse serum, 100 μg/ml of streptomycin and 100 U/ml of penicillin. The cells were split in a 1/6 ratio each week and the medium changed once during the week.

Cytotoxicity

Cytotoxic assays were performed as previously described [13] using PC12 cells grown in 24-well culture plates and labelled with $Na_2{}^{51}CrO_4$ (5 μCi/ml) for 24 h at 37°C. Cells were washed twice with fresh media and incubated for 60 min at 37°C in the presence of different concentrations of staph-α toxin. ^{51}Cr released from the cells was monitored by counting aliquots of the culture medium in a gamma scintillation counter. Total ^{51}Cr incorporation into PC12 cells was determined by measuring the release of radioactivity from cells solubilized with 0.5 N NaOH.

Arachidonic acid release

Staph-α toxin-stimulated release of arachidonic acid (AA) from isotopically labelled monolayer cultures of PC12 cells was measured following incubation with [^{3}H]-AA (0.1 μCi/ml) in serum-containing DMEM for 4 h at 37°C. After isotope incorporation, cells were washed twice with PBS, pH 7.4, that contained 1 mg/ml fatty acid-free BSA (Sigma, St. Louis). Fresh media was added and the cells were further incubated in the presence or absence of staph-α toxin at 37°C. Specific toxin concentrations and duration of incubation are indicated in the text of figure legends. At the appropriate time, medium containing released [^{3}H]-AA was collected and centrifuged at 800 *g* to remove residual cells. An aliquot of the supernatant was obtained and the amount of radioactivity present quantified by liquid scintillation spectrometry. Protein content of each sample was determined and the results expressed as cpm/mg. Basal, spontaneous release represents the amount of radioactivity detected in the media of untreated cultures after 30 min incubation.

Phosphoinositide assay

PC12 cells were pretreated with ^{3}H-inositol and assays performed as previously described [11]. Subsequently, the assays were stopped by addition of perchloric acid sufficient to yield a final acid concentration of 4.5% (vol/vol). Following a 30 min incubation on ice, samples were centrifuged at 3000 *g* for 5 min. The supernatant was then titrated to pH 8–9 with 0.5 M KOH/9 mM sodium borate. The samples were incubated on ice for an additional 20 min, then centrifuged at 3000 *g* for 5 min. The ^{3}H-inositol phosphates in the supernatants were isolated by anion exchange chromatography [11].

Intracellular calcium levels

The concentration of cytosolic free calcium was measured using the fluorescent calcium chelator Fura-2 [12], as previously described [14] with the following modifications. PC12 cells, maintained for three days as monolayer cultures, were collected following mechanical agitation, resuspended in their culture medium and incubated at 20°C for 60 min in the dark with 5 μM Fura-2/AM. Subsequently, the cells were washed three times in their culture medium and resuspended at a final density of 10^8 cells/ml. The fluorescence experiments were carried out at room temperature using a concentration of 2×10^6 cells/ml in a SPEX Fluorolog 2 spectrofluorimeter equipped with a stirred and thermostat-controlled cuvette holder. Intracellular conversion of the Fura-2/AM to Fura-2 was verified by running the excitation and emission spectra [25] ($_{ex}$ = 340 nm and $_{em}$ = 510 nm). A 435-nm cut-on filter was placed between the sample compartment and the emission detector to reduce light scattering. Bleaching of Fura-2 was minimized by reducing the excitation beam to 0.5 mm, while the slit width of the emission beam was set at 2.5 mm. Following the initial equilibration of fluorescent signal (which took about 3 min), the base line remained stable over the entire period of the experiment. To obtain the maximal fluorescence signal ($F_{infinite}$), the experiments were terminated by addition of 20 μM (Final concentration) of digitonin. F_o was measured after adding 25 μl aliquots of a mixture containing 0.5 M EDTA/1 M Tris/NaOH, pH 10.0.

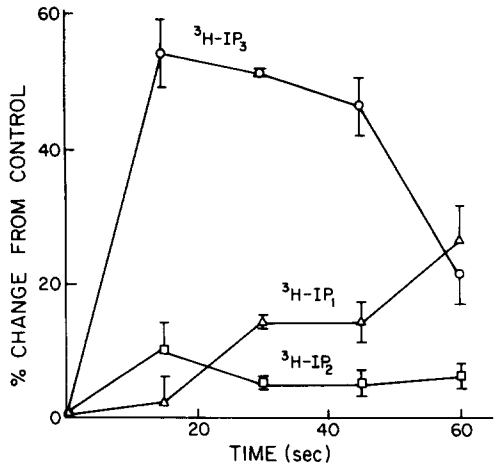

FIG. 1. Time course of staphylococcal α-toxin stimulated formation of ^{3}H-inositol phosphates. PC12 cells were preincubated with ^{3}H-inositol for 2 days and then incubated in phosphate saline buffer in the presence or absence of 70 nM α-toxin. At various times after toxin addition the formation of ^{3}H-inositol phosphates was determined. The results are expressed as percentage change from control ± standard deviations ($n=3$), where the control values are the level of ^{3}H-inositol phosphate in the absence of toxin at various times of incubation.

RESULTS

Staphylococcal α-toxin stimulation of phosphoinositide turnover

Staph-α toxin (70 nM) rapidly (15 s) stimulated formation of [^{3}H]IP$_3$, [^{3}H]IP$_2$ and [^{3}H]IP$_1$, with the greatest increase observed for [^{3}H]IP$_3$ (Fig. 1). Making the medium without calcium did not appear to affect the staph-α response. Since the changes were more apparent for [^{3}H]IP$_3$ (Fig. 1) we measured the effect of extracellular calcium on staph-α stimulation of this inositol phosphate. When PBS was added either with or without calcium then, 20 s after staph-α stimulation, [^{3}H]IP$_3$ ($n=3$) increased some 54 ± 5% or 58 ± 9% above control levels, respectively. In the absence of extracellular Ca^{2+} and presence of EGTA no stimulation by staph-α toxin of formation of inositol phosphates could be measured (data not shown), further indicating that extracellular calcium is required for stimulation of PhC activity in PC12 cells [11]. A second measure of phosphoinositide turnover, requiring a longer period of incubation (minutes), is the accumulation of [^{3}H]IP's in the presence of LiCl which is included to inhibit the breakdown of phosphorylated species to free inositol. This approach could not be utilized to characterize the effect of staph-α toxin on phosphoinositide hydrolysis due to cytotoxicity (data not shown). Enhanced staph-α cytotoxicity in the presence of LiCl could occur as a result of Li^+ influx through the staph-α toxin pores [15]. Formation of [^{3}H]IP$_3$ suggests that staph-α toxin induced hydrolysis of phosphoinositides is via a phospholipase C mediated pathway.

Staphylococcal α-toxin stimulation of arachidonic acid release

Staphylococcus aureus α-toxin stimulated the release of [^{3}H]-arachidonic acid (^{3}H-AA) from PC12 cells in a dose- and time-dependent fashion (Fig. 2). Enhanced release of [^{3}H]-AA elicited by staph-α occurred within the nanomolar concentration range [Fig. 2(A)]. The amount of radioactivity present in media increased 1.8-fold following a 60 min incubation with 125 nM staph-α, the highest subcytotoxic concentration tested [Fig. 2(A)]. At this concentration of staph-α, increased cytotoxicity was not microscopically visualized by the uptake of Trypan blue (data not shown). A nominal increase in cellular release of ^{51}Cr occurred. Following incubation for 60 min with 125 nM staph-α toxin a release of 25% of ^{51}Cr was measured. This amount of ^{51}Cr release represents a degree of toxicity well below one cytotoxic unit [13]. It appears that enhanced [^{3}H]-AA release was not a consequence of non-specific cytotoxicity but may be correlated with staph-α-induced changes in membrane permeability.

Stimulation of [^{3}H]-AA release by staph-α is time-dependent [Fig. 2(B)]. Addition of 70 nM staph-α to PC12 cells caused a 25% increase in [^{3}H]-AA release within 1 min. The amount of this initial [^{3}H]-AA release did not change up to 10 min, after which there was a sharp rise in staph-α-stimulated [^{3}H]-AA release. At 30 min,

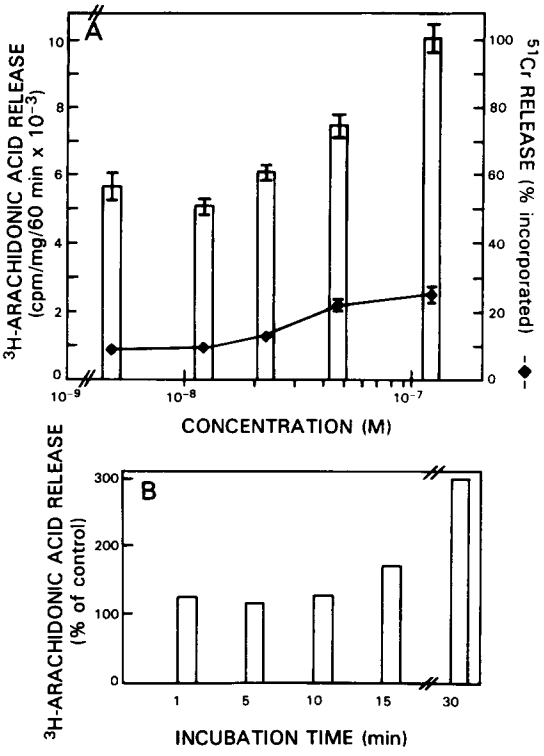

FIG. 2. Dose- and time-dependent effects of staphylococcal α-toxin on cytotoxicity and release of arachidonic acid from PC12 cells. Panel A—PC12 cells pretreated with [^{3}H]-AA or ^{51}Cr were incubated with 4.7, 11.8, 23.6, 47.2 or 118.0 nM staph-α at 37°C for 60 min. Release of radioactivity was measured as described in Materials and Methods. The open bars represent [^{3}H]-AA release (cpm/mg/60 min). Closed symbols represent ^{51}Cr release (percentage of total incorporated). Values are expressed as the mean ± S.E.M. for measurements performed in triplicate. Spontaneous release of [^{3}H]-AA from PC12 cells was 5576 ± 276 cpm/mg/60 min. Panel B—PC12 cells, isotopically labelled with [^{3}H]-AA, were incubated with 70 nM staph-α for 1, 5, 10, 15 or 30 min at 37°C. The results, expressed as a percentage of control: (cpm/mg evoked release)/(cpm/mg spontaneous release) represent the mean of duplicate determinations.

release of [^{3}H]-AA elicited by 70 nM staph-α reached levels that were 304% of control and the cytotoxicity was 5–10%.

When Ca^{2+} was left out of the incubation buffer, then the staph-α (70 nM)-mediated stimulation of [^{3}H]-AA release was diminished by some 70% (60 min incubation). However, the removal of calcium and addition of EGTA resulted in cell monolayer detachment and a very high variability of PhA_2 measurements (data not shown), preventing a reliable estimation. High pressure liquid chromatography and radioimmunoassay identified the prostaglandins PGE_2 and PGD_2 [25] as the major products of [^{3}H] arachidonic acid metabolites released by PC12 cells treated with 70 nM staph-α for 30 min (Goldin E., personal communication).

Effect of staph-α toxin on intracellular calcium level

One plausible explanation for stimulation of PC12 phospholipases by staph-α toxin could be disruption of cellular calcium homeostasis. Measurements of the intracellular free Ca^{2+} concentrations using Fura-2 confirmed this possibility, as shown in Fig. 3. The basal level of $[Ca^{2+}]_{in}$ in the 3 day cultures of undifferentiated PC12 cells was found to be approximately 95 ± 15 nM ($n = 5$). Stimulation with the cholinergic agonist, carbachol, rapidly and transiently increased $[Ca^{2+}]_{in}$ to a peak value of about 340 nM [Fig. 3(A)] as previously reported [16]. The fluorescence response of Fura-2 loaded PC12 cells following stimulation with 50 mM potassium differed from the response observed subsequent to carbachol addition. In the presence of high K^+, the $[Ca^{2+}]_{in}$ increased rapidly to a value of 405 ± 20 nM [Fig. 3(A)], then gradually declined over an extended period of time, as expected [16]. Exposure of PC12 cells to 300 nM staph-α toxin had a dual effect on the change in $[Ca^{2+}]_{in}$: addition of staph-α caused an immediate, 2-fold increase in $[Ca^{2+}]_{in}$ reaching an initial plateau within 2–3 min [Fig. 3(A or C)]. From this initial plateau, $[Ca^{2+}]_{in}$ gradually increased to 600–800 nM within 7–10 min following staph-α toxin addition [Fig. 3(A)]. Subsequent treatment with carbachol or K^+ depolarization of PC12 cells treated for 7 min with 300 nM staph-α toxin either failed to increase further $[Ca^{2+}]_{in}$ or had a modest additive effect. The rate of staph-α toxin-induced increase in $[Ca^{2+}]_{in}$ was found to be dose-dependent [Fig. 3(B)]: Low staph α-

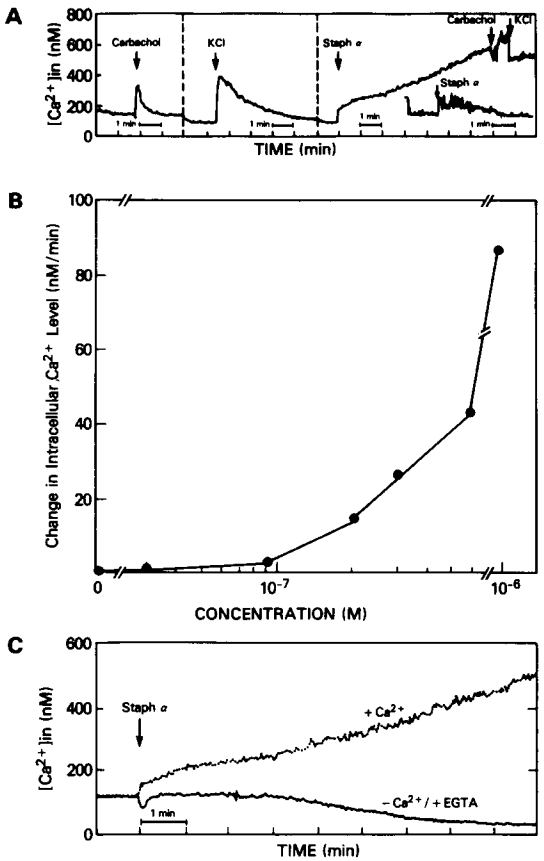

FIG. 3. The effect of staphylococcal α-toxin on intracellular Ca^{2+} concentration in PC12 cells. (A) Left—Control response of cells stimulated with 100 μM carbachol. Middle—Control response to depolarization with 50 mM KCl. Right—The effect of 300 nM staphylococcal α-toxin; following the slow and prolonged increase in $[Ca^{2+}]_{in}$, addition of 100 μM carbachol still evoked a small increase in intracellular calcium; subsequent addition of KCl resulted in a slight drop in the signal, probably due to a dilution effect. Inset—Addition of a subcytotoxic concentration of staphylococcal α-toxin results in a transient, oscillatory increase of $[Ca^{2+}]_{in}$. (B) Dose response of the rate of increase in $[Ca^{2+}]_{in}$. The data points represent the slopes of the Fura-2 fluorescence signals, taken one and three minutes following addition of the respective α-toxin concentrations. (C) Effect of extracellular Ca^{2+} on staphylococcus α-toxin induced increase in $[Ca^{2+}]_{in}$. The experiments were performed in Krebs–Ringer with 2.5 mM calcium (upper trace) or with 2.5 mM EGTA (lower trace). At the arrow, 250 nM staph-α toxin was added. The decrease in $[Ca^{2+}]_{in}$ in the presence of EGTA, independent of toxin presence, probably reflects the draining of intracellular calcium.

toxin concentrations (40 nM) stimulated a transient, oscillating 2 min increase in $[Ca^{2+}]_{in}$ [Fig. 3(A), right panel inset]. At subcytotoxic concentrations (10^{-8}–10^{-7} M) there was a minimal change in intracellular Ca^{2+} [Fig. 3(B)]; at 1×10^{-7}–5×10^{-7} M subcytotoxic concentrations of staph-α toxin the rate of $[Ca^{2+}]_{in}$ change increased from 5 nM/min (1×10^{-7} M) to 45 nM/min (5×10^{-7} M) [Fig. 3(B)]. At 10^{-6} M staph-α toxin caused cytotoxicity, membrane leakage (data not shown) and a substantial increase in $[Ca^{2+}]_{in}$ [Fig. 3(B)].

The increase in $[Ca^{2+}]_{in}$ mediated by staph-α toxin could be due to either Ca^{2+} influx or release from intracellular stores. To determine the source of Ca^{2+} responsible for staph-α-induced increases in $[Ca^{2+}]_{in}$ experiments were performed, in the presence or absence of extracellular Ca^{2+} [Fig. 3(C)]. Staph-α toxin induced a fluorescence response of the Fura-2 loaded PC12 cells only in the presence of extracellular Ca^{2+} [Fig. 3(C)]. These results suggest staph α-toxin mediated a Ca^{2+} influx, but did not induce release of Ca^{2+} from intracellular stores.

DISCUSSION

Addition of subcytotoxic concentrations of staph-α toxin (10^{-8}–10^{-7} M) to undifferentiated PC12 cell cultures elicited stimulation of PI hydrolysis and arachidonic acid release within 15 s and 10 min, respectively. These observations suggest staph-α toxin mediates activation of phospholipase C and A_2. Enzymatic activations occurred without evidence of gross disruption of cell surface membrane integrity or cytotoxicity. Within the same time course of toxin-mediated enzyme activation a biphasic effect of staph-α toxin (10^{-7}–10^{-6} M) on the intracellular calcium was measured. The first phase was composed of a rapid 2-fold rise in $[Ca^{2+}]_{in}$ concentration within 1 min. The initial $[Ca^{2+}]$ increase was followed by a 10 min progressive influx of $[Ca^{2+}]$.

A plausible explanation for these observations is that initial binding of staph-α toxin to the PC12 cell membranes modifies membrane organization [3, 17] resulting in a rapid acti-

vation of phospholipase C, formation of IP_3 and subsequent release of Ca^{2+} from intracellular stores. This metabolic pathway may be responsible for the early, small $[Ca^{2+}]_{in}$ rise measured. Staph-α activation of PhC appears to be independent of extracellular calcium, therefore suggesting another cellular signal for activation of this enzyme. The absence of a staph-α effect on $[Ca^{2+}]_{in}$ when Ca^{2+} is removed from the extracellular media and EGTA is added, could alternatively be explained by an absolute requirement of PC12 cells phospholipase C on membrane associated Ca^{2+} for activation [11]. The possibility that the initial rise in intracellular Ca^{2+} caused by staph-α toxin is due to pore formation and not activation of PhC cannot be discounted but seems unlikely when considering the length of time required for organization of staph-α pore structures [2, 3]. The delayed second phase of gradual Ca^{2+} influx caused by staph-α toxin could be mediated by pores that form in PC12 cell membranes [2, 3]. The secondary rise in $[Ca^{2+}]_{in}$ is most probably responsible for the delayed activation of phospholipase A_2, resulting in release of arachidonic acid. Indeed the dependency of PhA_2 activation on extracellular Ca^{2+} further supports this possibility. Alternatively, arachidonic acid could be generated from phosphoinositides by the action of phospholipase C and diglyceride lipase [18] but this pathway seems less likely to be responsible for AA formation since this lipase is not as sensitive to $[Ca^{2+}]_{in}$ as PhA_2, with regard to activation [19].

Another point to be considered is that activation of these phospholipases by staph-α toxin is not receptor mediated [19]. High affinity binding sites for staph-α toxin have not been detected in eukaryotic cell membranes [20]. It was reported that the staph-α toxin has a specific affinity for *N*-acetyl-glucosamine-containing gangliosides [21] which are present in PC12 cell membranes [22] and, therefore, might be involved in the binding and insertion of the toxin into the membrane prior to the activation of the phospholipases and initiation of the Ca^{2+} influx.

Recently, it was proposed that staph-α toxin could serve as a suitable tool for controlled and selective plasma membrane permeabilization, therefore enabling the study of intracellular actions of hormones and drugs [2]. The minimal requirements for exocytosis [5] and the calcium-triggered dopamine release from staph-α toxin permeabilized PC12 cells [23] have been characterized and it has been proposed that staph-α toxin permeabilization represents a suitable method for studying events associated with exocytosis in secretory cells. In this study, we present evidence that phospholipases and $[Ca^{2+}]$ homeostasis, which play key roles in secretion were activated by staph-α toxin at subcytotoxic, permeabilizing concentrations; therefore the usage of this toxin as a permeabilizing tool in cell biology should be re-evaluated.

Finally, it should be emphasized that activation of cellular phospholipases and the Ca^{2+} influx signal induced by staph-α toxin could explain a long series of pathological effects induced by this toxin such as disruption of myelin [8], reduction in EGF receptor affinity [13], neurotoxicity and the steps leading to cell death [24].

Acknowledgements—We gratefully acknowledge Dr GORDON GUROFF, Section on Growth Factors and Dr HARVEY POLLARD, Laboratory of Cell Biology, NIH for their encouragement in the pursuit of this study. The typing of the manuscript by Mrs JUDITH GOLDSTEIN is appreciated.

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