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CHOLERA TOXIN AFFECTS NUCLEAR ADP-RIBOSYLATION IN GH1 CELLS

Ana Aranda*, Angel Pascual*, Richard Copp+ and Herbert Samuels+

+Division of Molecular Endocrinology, The Rose F. Tishman Laboratories for Geriatric Endocrinology. Department of Medicine, New York University Medical Center, New York 10016; and *Unidad de Endocrinologia Experimental, Instituto de Investigaciones Biomedicas. CSIC. Fctad de Medicina. Universidad Autonoma. 28029 Madrid. Spain

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SUMMARY Incubation of GH1 cells with cholera toxin for 24 h inhibits [^{32}P]ADP-ribose incorporation into histones and non-histone nuclear proteins by more than 50%. The toxin produces a generalized decrease of incorporation into all protein acceptors and into the poly(ADP-ribosyl)ated components excised from chromatin after micrococcal nuclease digestion. The cellular levels of NAD were also decreased (40 to 80%) after treatment with cholera toxin. The inhibition of poly(ADP-ribosyl)ation is preceeded by an increase of [^{32}P]ADP-ribose incorporation, since incubation with the toxin for 3 h caused an increase instead of a decrease of incorporation. Incubation with dibutyryl cyclic AMP for 24 h also inhibited nuclear poly(ADP-ribosyl)ation, thus showing that the effect of cholera toxin might be mediated by cyclic AMP.

Covalent modification of proteins by ADP-ribosylation modulates a variety of cellular responses (1,2). Poly(ADP-ribosylation) of nuclear proteins has been implicated in cell differentiation and transformation, gene expression, and DNA replication and repair, all of which might involve changes in the structure and function of chromatin (for review see 1,2).

Cholera toxin (ChT) enhances adenylate cyclase activity by catalyzing ADP-ribosylation of the stimulatory guanine nucleotide binding protein of the cyclase (3). Although most of the cellular effects of the toxin are directly or indirectly caused by increases in intracellular cyclic AMP (cAMP) levels, recent studies have also shown that some of the effects of ChT appear to be mediated by cAMP-independent mechanisms (4-6). This raises the possibility that internalization of the catalytic subunit of the toxin may modify cellular functions other than the adenylate cyclase system. In this study we examined whether or not ChT alters the poly(ADP-ribosyl)ation of chromatin proteins. Using pituitary GH1 cells we report that the toxin can influence poly(ADP-ribosyl)ation of histones and non-histone chromatin proteins and that this effect is, at least partially, due to cyclic nucleotide regulation.

MATERIALS AND METHODS

ADP-ribosylation assay. GH1 cells were grown in monolayers as previously described (4,7). The nuclei were isolated (7) and incubated for 15 min at 37ºC in 0.5 ml of 0.1 M Tris-HCl pH 7.9, 2 mM MgCl2, 1 mM dithiothreitol, containing 5-10 µCi adenylate-[^{32}P]NAD ([^{32}P]NAD), SA 30-40 Ci/mmol. After chilling the nuclei were pelleted and extensively washed with the same buffer. Histones were

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extracted with 0.4 N sulfuric acid, precipitated with acetone (7) and dissolved in 0.9 N glacial acetic acid. The remaining nuclear pellet, containing non-histone proteins, was precipitated overnight with 3 ml ethanol at -20º C. After centrifugation at 3,000 x g x 45 min, the pellets were dissolved in 10 mM sodium phosphate , 1% sodium dodecyl sulphate (SDS), pH 7.4. An aliquot of the histone and non-histone proteins fractions was used to analyze incorporation by Cerenkov radiation, and the same sample used for the determination of protein content by the method of Lowry (8). Another aliquot was saved for gel electrophoresis

Gel electrophoresis. Non-histone proteins were analyzed by SDS-polyacrylamide gradient (5-15%) gel electrophoresis by the method of Laemmli (9). Histones were electrophoresed in 15% polyacrylamide gels in the presence of 0.9% acetic acid and 2.5 M urea as described by Panyim and Chalkley (10). After electrophoresis the gels were stained, destained and autoradiographed.

Micrococcal nuclease digestion. GH1 cells nuclei were incubated with 50 μCi [^{32}P]NAD/50 A260 nm of nuclear material for 15 min at 37ºC. The nuclei were washed three times with a buffer containing 10 mM Tris-HCl pH 7.4, 1 mM CaCl2 , 5 mM 2-mercaptoethanol, and 20 U/ml Trasylol, and digested at 0ºC for 30 min in 0.5 ml of the same buffer containing 15 units of micrococcal nuclease/A260 nm units of nuclei. The mixture was then centrifuged at 3,000 x g x 10 min and the supernatant saved and made 5 mM with EDTA to stop further digestion. The supernatant fractions were centrifuged for 18 h at 36,000 rpm in isokinetic sucrose gradients and separated into approximately 35 fractions as previously described (7). Trichloroacetic acid (TCA) was added to each fraction to achieve a final concentration of 25% (w/v) and the TCA-insoluble fraction used for determination of radioactivity and DNA (11) content.

cAMP determination. Intracellular and medium cAMP levels were determined as previously described (4). The samples were acetylated and cAMP determined by radioimmunoassay using the reagents provided by New England Nuclear.

NAD determination. Cellular NAD levels were determined spectrophotometrically (12) in 0.5 N perchloric acid extracts previously neutralized to pH 7.4 with 1N K_2PO_4 and 3 N KOH.

RESULTS AND DISCUSSION

Fig.1 shows the effect of a 24-h incubation with ChT on [^{32}P]ADP-ribose incorporation into histones and non-histone proteins. While ChT 0.1 nM reduced incorporation by 30-40% into both fractions, 30 nM ChT inhibited incorporation by 50-60%. The latter was almost maximal, since in additional experiments ChT 50 to100 nM produced a 50-70% decrease. Furthermore, the intact toxin was necessary for this effect since incubation of GH1 cells with 100 nM of the B protomer of the toxin did not alter ADP-ribosylation (not shown).

The protein acceptors of [^{32}P]ADP-ribose among the histones are illustrated in Fig.2. The strongest labelled band was histone H1 which is also labelled after incubation of nuclei from other cell types with [^{32}P]NAD (1). In contrast with other systems where H2B is an important histone

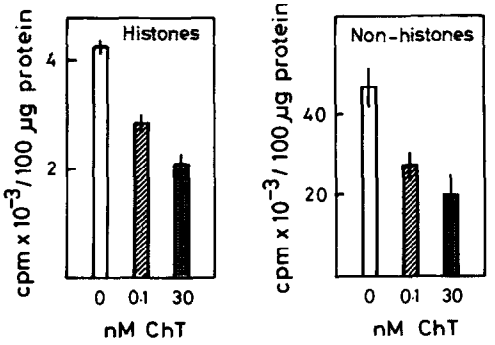

FIGURE 1. Effect of cholera toxin on [^{32}P]ADP-ribose incorporation. Triplicate flaks of GH1 cells were incubated for 24 h with cholera toxin. The nuclei were isolated, incubated with [^{32}P]NAD, and the radioactivity associated with histone and non-histone proteins determined.

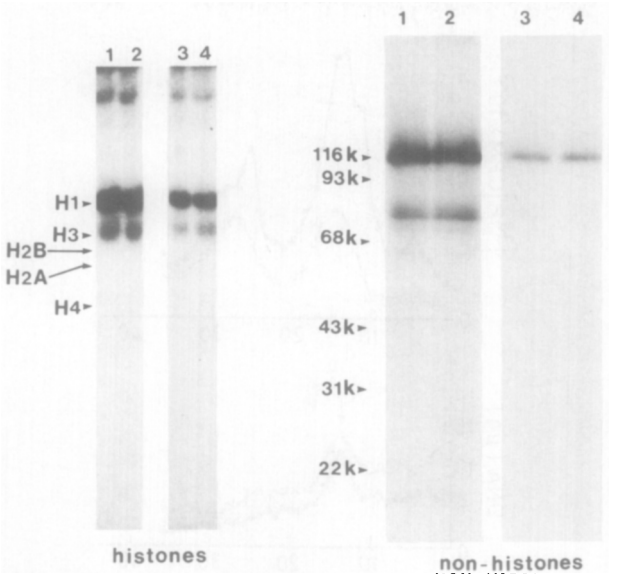

FIGURE 2. Autoradiograms of poly(ADP-ribosyl)ated nuclear proteins. GH1 cells were incubated with (lanes 3 and 4) or whithout (lanes 1 and 2) 60 nM ChT for 24 h. The gels corresponding to histones (left) and non-histone proteins (right) were stained, destained and autoradiographed. The identity of the radioactive bands was determined by comparison with the migration of the stained bands (histones) or with the migration of the following molecular weight standards (non-histones): soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (92,500), ß-galactosidase (116,000).

acceptor (13), in GH1 cells other radioactive band migrated identically to the stained band corresponding to histone H3. Other fainter radioactive bands migrating slower than H1 were also observed. These bands could represent HMG proteins which have been shown to be ADP-ribosylated in other cells (14). The same proteins were labelled in control cells and in cells incubated with ChT, although in agreement with the data presented in Fig.1, incubation with the toxin reduced labelling of all bands. Among the non-histones the main acceptor corresponded to a band with an Mr of 110,000-120,000. This protein probably represents the enzyme poly(ADP-ribose)synthetase which has been described to undergo extensive automodification and to incorporate most of the poly(ADP-ribose) in isolated nuclei (15). Other radiolabelled non-histone proteins ranging from 60,000 to 90,000 Mr were also observed. As with the histones, ChT equally decreased incorporation into the different protein acceptors, suggesting that the toxin produces a non-selective inhibition of poly(ADP-ribosyl)ation. This is further demonstrated by the experiment illustrated in Fig.3 in which the sedimentation profile of the [^{32}P]ADP-ribosylated components excised from chromatin after digestion with micrococcal nuclease was examined. It has been described that poly(ADP-ribose)synthetase activity is preferentially associated with the fraction of chromatin more sensitive to nucleases (16). After digestion of GH1 cells nuclei most of the [^{32}P]ADP-ribose sedimented with the mononucleosome particles at a 11.5 S peak and other less prominent peak sedimented at the 4 S region of the gradient. Incubation with ChT inhibited the extent of incorporation but did not alter the sedimentation profile.

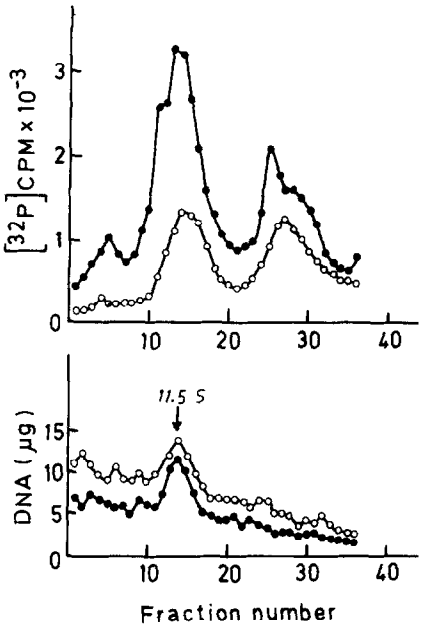

FIGURE 3. Micrococcal nuclease digestion of poly(ADP-ribosyl)ated chromatin. The cells were incubated for 24 h with (0) or without (0) 60 nM ChT. The nuclei were isolated, incubated with [^{32}P]NAD and digested. The released material was sedimented in isokinetic sucrose gradients, and the radioactivity (upper panel) and DNA (lower panel) content of each fraction determined.

The generalized effect of ChT on the different protein acceptors and chromatin components suggests that its effect could be secondary to a decrease in the activity of the enzyme or to a change in the availability of substrate. Since stimulation of ADP-ribosylation by poly(ADP-ribose)synthetase leads to self-inhibition of the enzyme through auto-modification (17), there was the possibility that an initial stimulation of poly(ADP-ribosyl)ation by ChT could result in the subsequent decrease found at 24 h. Table 1 shows that ChT stimulated [^{32}P]ADP-ribose incorporation into nuclear proteins during the first 3.5 h, and decreased it at 24 h. Additionally, ChT elicited a 4-fold decrease in cellular NAD levels after 2 h and 5 h of incubation (from 2.3 nmol/mg protein in control cells to 0.5-0.6 nmol/mg protein in cells

TABLE 1. Time-course of the effect of cholera toxin

	[^{32}P]ADP-ribose incorporation (cpm/100 μg protein)			
	Time of incubation (hours)			
	0	1.5	3.5	24
Histones	2,077 ± 256	2,858 ± 428	3,977 ± 438	982 ± 76
Non-histones	9,737 ± 1,088	12,501 ± 2,135	14,894 ± 1,107	5,259 ± 108

GH1 cells were incubated with 60 nM cholera toxin for the times indicated. The nuclei were incubated with [^{32}P]NAD and the radioactivity incorporated into histones and non-histone proteins determined. Data are mean ± SD of triplicate cultures.

TABLE 2. Effect of cholera toxin on cAMP production

Cholera toxin (nM)	cAMP (pmol/culture)	
	Intracellular	Medium
0	1 ± 0	10 ± 1
0.1	22 ± 3	2,164 ± 198
60	36 ± 4	571 ± 85

cAMP levels were determined after 24 h incubation with the concentrations of cholera toxin indicated. Data are mean ± SD of triplicate cultures.

incubated with 60 nM ChT). After 24 h, NAD levels were still significantly reduced (1.0 nmol/mg protein) with respect to those found in control cells. The reduction in NAD levels is consistent with the enhanced utilization resulting from the effect of ChT on stimulating ADP-ribosylation of membrane proteins (18). Therefore, the stimulation of [^{32}P]ADP-ribose incorporation into nuclear proteins found during the first hours of incubation with ChT may reflect an increase in the specific activity of the [^{32}P]NAD in the *in vitro* incubation compared to control cells. By contrast, this does not provide an explanation for the inhibition of poly(ADP-ribosyl)ation found at 24 h, since an inhibition of [^{32}P]ADP-ribose incorporation into the nuclei of ChT-treated cells at this time was accompanied by a significant decrease of cellular NAD levels.

Table 2 shows the influence of a 24-h incubation with 0.1 nM and 50 nM ChT on cAMP levels in the medium and the cells. In contrast with the data obtained for [^{32}P]ADP-ribose incorporation, 0.1 nM ChT elicited a greater cAMP response than that produced by a higher concentration of ChT (50 nM). This biphasic effect of the toxin had been previously observed by us in GH1 cells (4). The finding that the effect on poly(ADP-ribosyl)ation did not correlate with the stimulation of cAMP suggested that the influence of ChT on poly(ADP-ribosyl)ation could be mediated by a cAMP-independent mechanism. To test this possibility we examined the influence of dibutyryl cAMP (db-cAMP) on poly(ADP-ribosyl)ation of histones and non-histone proteins. As shown in Table 3, a 24-h incubation with 1 mM db-cAMP also elicited a decrease of [^{32}P]ADP-ribose incorporation into both fractions. Since this cAMP analog contains butyrate which influences nuclear ADP-ribosylation in other cells (19), we compared the effect of db-cAMP with that

TABLE 3. Effect of cyclic AMP on [^{32}P]ADP-ribose incorporation

	cpm/100 µg protein	
	HISTONES	NON-HISTONES
Control	1,456 ± 145	21,046 ± 1,077
ChT 60 nM	729 ± 75	11,509 ± 783
db-cAMP 1 mM	603 ± 42	7,777 ± 751
butyrate 2 mM	889 ± 87	12,788 ± 1,720

The cells were incubated with the concentrations of the compounds indicated for 24 h. The data are mean ± SD of three separate cultures.

produced by butyrate. This compound also inhibited [^{32}P]ADP-ribose incorporation, suggesting that the effect of db-cAMP could be, to a certain extent, attributed to the butyrate released. However, the effect of both compounds was quantitatively different and while 1 mM db-cAMP decreased incorporation by 60%, a 2 mM concentration of butyrate only reduced incorporation by 40%. This shows that cAMP *per se* decreases poly(ADP-ribosyl)ation in GH1 cells and that, therefore, the effect of ChT can be, at least partially, due to cAMP production.

cAMP is implicated in cell differentiation and in the regulation of gene expression in eukaryotes. It has been proposed that, in addition to the existence of cAMP regulatory elements within the genes (20), cAMP alters transcription by changing phosphorylation of chromosomal proteins (21). In the present study we also demonstrate that cAMP might alter other post-synthetic modification of nuclear proteins, i.e poly(ADP-ribosyl)ation, which also has regulatory effects on chromatin structure and, as a consequence, on gene expression.

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