

NSL 08284

## Activation of locus coeruleus (LC) neurones by cholera toxin: mediation by cAMP-dependent protein kinase

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(Received 7 August 1991; Revised version received 17 September 1991; Accepted 17 September 1991)

**Key words:** Cholera toxin; Adenylate cyclase; cAMP; Whole cell; Pacemaker; Locus ceruleus; Protein kinase inhibitor; Patch clamp; Noradrenergic

There is evidence that the tonic pacemaker activity of the noradrenergic pacemaker neurones of the locus coeruleus (LC) depends on endogenous cAMP acting via protein kinase A and its phosphorylation pathway. In this study, we tested the effect of cholera toxin, which produces persistent activation of  $G_s$ , on LC firing rates. Bath applied cholera toxin (holotoxin) increased LC firing rates after a lag of 50–110 min. Intracellularly applied A-subunit (*active*-subunit) but not the B-subunit (*binding*-subunit) of cholera toxin via low resistance patch electrodes mimicked the excitatory actions of bath-applied holotoxin but without its lag period. The effects of both bath-applied and intracellularly applied cholera toxin A-subunit were blocked by intracellular applications of a specific cAMP-dependent protein kinase inhibitor (PKI<sub>5-24</sub>). We conclude that persistent activation of  $G_s$  by cholera toxin (A-subunit) increases LC firing rates via the cAMP-dependent protein phosphorylation pathway.

Noradrenergic neurones of the rat locus coeruleus (LC) are endogenous pacemakers that exhibit slow, tonic firing even in the absence of synaptic inputs [3, 15, 17, 25]. We previously reported that LC pacemaker activity is dependent on a diffusible intracellular molecule based on the observation that a large proportion of LC neurones stop firing on establishing whole cell recording with low resistance patch electrodes [2]. This washout effect was enhanced by intracellular applications of a specific cAMP-dependent protein kinase inhibitor (PKI<sub>5-24</sub>). In contrast, elevation of intracellular levels of cAMP or the catalytic subunit of protein kinase A prevented the loss of firing and increased LC firing rates in a dose-dependent manner. These excitations were blocked by intracellular PKI<sub>5-24</sub>, suggesting the involvement of endogenous cAMP and its protein phosphorylation pathway in LC pacemaker mechanisms.

In the present study, we studied the effect of cholera toxin on LC pacemaker activity. Cholera toxin, which is a bacterial exotoxin secreted by *Vibrio cholerae*, ADP-ribosylates the G protein [5]  $G_s$  and thereby stimulates adenylate cyclase activity in a variety of tissues in an apparently irreversible manner [6, 7, 10], thus elevating intracellular cAMP levels. The holotoxin is comprised of

two subunits, A (active) [9, 13] which cannot cross the cell membrane on its own, and B (binding) which has no toxic action but is required for translocation of the toxin across the cell membrane [10, 13, 14]. We tested the effect of extracellular applications of the holotoxin cholera toxin on LC firing rates. In addition, we used the whole-cell mode of patch-clamping to introduce the A- or B-subunits of cholera toxin into individual cells, and studied their effect on LC firing rates. We also tested the effect of intracellular applications of PKI<sub>5-24</sub> on the actions of both extracellularly and intracellularly applied cholera toxin.

LC slices were prepared as previously described [1]. In brief, 500  $\mu$ m thick slices were incubated at  $33 \pm 0.5^\circ\text{C}$  in an interface chamber continuously perfused with artificial cerebrospinal fluid (ACSF) at a rate of  $\sim 1$  ml/min. The ACSF (pH 7.35–7.38) equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  contained (in mM): NaCl 126, KCl 5,  $\text{NaH}_2\text{PO}_4$  1.25, D-glucose 10,  $\text{NaHCO}_3$  25,  $\text{CaCl}_2$  2 and  $\text{MgSO}_4$  2. The LCs were visually identified in rat brainstem slices as dark oval areas in the upper pons on the lateral borders of the central grey and the fourth ventricle, at a frontal plane at or just anterior to the genu of the facial nerve. Cholera toxin and its subunits were obtained from List and PKI<sub>5-24</sub> from Peninsula.

The effect of bath applications of cholera toxin on spontaneous firing rates of LC neurones was studied using extracellular recording techniques. Blunt-tipped

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electrodes filled with 150 mM NaCl (3–5 M $\Omega$ ) were used to record extracellular potentials through a high input impedance amplifier. Noradrenergic neurones were identified by their triphasic waveform, regular rhythm and firing rates of less than 4 Hz [3]. Following bath application of the holotoxin, firing rates were monitored for periods of 2–4 h. The involvement of the cAMP-dependent phosphorylation pathway in the action of bath-applied cholera toxin was tested later by dialyzing LC neurones in the toxin-treated slice with PKI<sub>5–24</sub>.

The whole-cell mode of patch-clamping [12] was used, both to introduce PKI<sub>5–24</sub> into individual cells and to study its effect on LC firing rates. Whole-cell recordings in brain slices [2, 4] were carried out using an Axoclamp-2A amplifier. Pipette access to the cell's interior ( $R_a$ ) was continually monitored through the bridge circuit by recording the instantaneous voltage responses to 200 pA  $\times$  200 ms pulses. The control pipette solution contained (in mM): K gluconate 120, HEPES 10, BAPTA K<sub>4</sub> 5, sucrose 20, CaCl<sub>2</sub> 2.38, MgCl<sub>2</sub> 1, K<sub>2</sub>ATP 1 and GTP 0.1. The free Ca<sup>2+</sup> concentration in the control pipette solution was computed to be 97 nM, assuming an absolute affinity constant for BAPTA of  $9.35 \times 10^6$  M<sup>-1</sup> and pH independence [21]. PKI<sub>5–24</sub> (100  $\mu$ M) was added to the control pipette solution. The pH was adjusted to 7.32–7.35 with KOH. Following formation of a giga-seal with a patch pipette (electrode resistance, 2.0–3.0 M $\Omega$ ) the basal frequency of firing was recorded extracellularly, in the cell-attached mode. To assess the effect of intracellular dialysis with control and test pipette solutions, the basal firing rates were first recorded in the cell-attached and/or *partial access* (pa) modes. The term *partial access* refers to the mode where there is incomplete rupturing of the membrane patch; it is characterized by a high series resistance relative to electrode resistance ( $R_a > 6$  M $\Omega$ ), truncated spikes, and a high apparent input resistance [2]. These rates were compared with those obtained after rupturing the patch with additional suction to establish full whole-cell access; the latter is defined as the condition in which the series resistance approaches the electrode resistance (access resistance ( $R_a$ ) = 3–6 M $\Omega$ ).

The effect of intracellular applications of the A- or B-subunits of cholera toxin on LC firing rates was similarly studied using low-resistance patch electrodes and the whole-cell configuration. The effect of intracellular application of A-subunit (CTX-A) was tested after reduction with dithiothreitol (DTT) which reduces the disulphide bond between the A1 and A2 moieties of the A-subunit [16] and releases the active moiety, A1 [10, 19]. The B-subunit (CTX-B) was tested both with and without prior reduction. Reduction was carried out by incubating the toxin subunits with DTT resin beads (Calbiochem, Reductacryl) for 30 min at room temperature. In

this resin form, DTT is immobilized on a polyacrylamide matrix so that no soluble components are added to the system. The DTT beads were later removed by filtration (Millipore, 0.22  $\mu$ m) and the subunits diluted to the desired concentration with the control pipette solution. NAD (100  $\mu$ M) which is an essential co-factor for cholera toxin action [8, 18] was included in patch-pipettes containing the toxin subunits. The effect of PKI<sub>5–24</sub> (100  $\mu$ M) on cholera toxin subunit action was studied by adding it to the pipette solution.

Basal firing rates of 0.3–1.5 Hz ( $0.82 \pm 0.2$ ;  $n = 5$ ), similar to those previously reported [2, 3, 11, 23], were recorded extracellularly from LC neurones prior to treatment with cholera toxin. In these 5 cells bath applications of the holotoxin form of cholera toxin (40–100  $\mu$ g/ml) for 8–35 min were followed by a delayed, gradual, and long-lasting increase in basal firing rates (Fig. 1). The lag period for a 100% increase in basal firing rates was 50–110 min. A maximal increase of 500–600% in basal firing rates was seen 80–180 min from the beginning of the application period. In the dose ranges tested, the length of the lag period was not affected appreciably by either the dose or duration of cholera toxin application.

Two hours following bath application of cholera toxin, whole cell recordings were performed in the toxin-treated slice with low-resistance patch electrodes containing PKI<sub>5–24</sub>. In 4 cells basal firing rates of 2.6–3.4 Hz were recorded in the cell-attached mode prior to intracellular dialysis with PKI<sub>5–24</sub>. Because of the pretreatment with cholera toxin these rates were significantly higher than those obtained prior to toxin treatment ( $3.1 \pm 0.19$

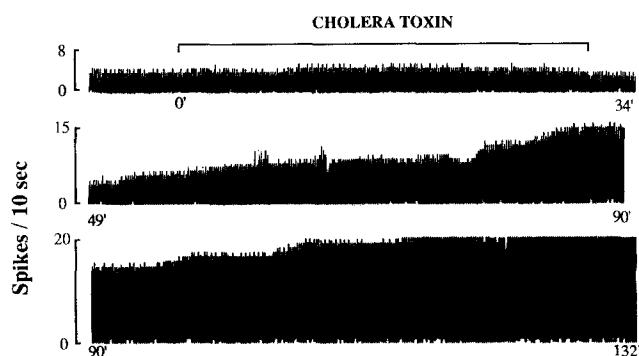


Fig. 1. Extracellular recording showing the effect of bath-applied cholera toxin (50  $\mu$ g/ml) on the spontaneous firing rate of an LC neurone. The cell had a basal firing rate of 0.6 Hz. Cholera toxin (50  $\mu$ g/ml) applied for 32 min produced a very gradual increase in LC firing rate. A 100% increase in firing rate was recorded after a lag period of 75 min and a maximal increase of 500% in the basal firing rate was seen at 150 min (out of recording range). No decline in firing rate was observed up to the end of the recording session (250 min following application of the holotoxin).

Hz as compared to  $0.82 \pm 0.2$  in above controls ( $P < 0.001$ ; *t*-test). Intracellular applications of PKI<sub>5-24</sub> decreased the elevated firing rates produced by bath applications of the holotoxin (Fig. 2) to near control values ( $0.88 \pm 0.52$ ;  $n = 4$ ). In contrast, intracellular dialysis with control pipette solution was followed by an overall increase in firing ( $n = 3$ ). The increased firing in these cholera toxin-pretreated cells may be attributed to the enhanced adenylate cyclase activity caused by a sudden increase in the availability of GTP (which is included in all the pipette solutions) on establishing whole-cell recording. Addition of the same concentration of GTP (100  $\mu$ M) to cholera toxin-treated erythrocyte membranes has been shown to significantly enhance adenylate cyclase activity [5].

Since bath applications of the holotoxin increased LC firing rates, we tested the effect of intracellular applications of the A- and B-subunits of cholera toxin on LC pacemaker activity using low-resistance patch electrodes. Basal firing rates of 0.7–2.3 Hz were recorded in cell-attached and/or partial access modes. As observed in an earlier study [2] a large proportion of cells stopped firing within 10–240 s of establishing whole-cell recording.

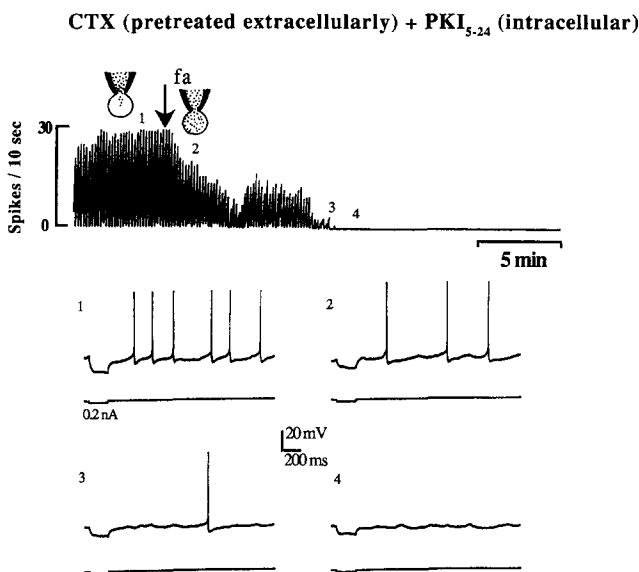


Fig. 2. Whole-cell recording showing the effect of intracellularly applied PKI<sub>5-24</sub> on the basal firing rate of an LC neurone recorded in a brain slice pretreated with cholera toxin. The holotoxin (50  $\mu$ g/ml) had been bath applied for 32 min and whole-cell recording was performed 2 h later. This cell had a basal firing rate of 3 Hz recorded after incomplete rupture of the patch ( $R_a > 8$  M $\Omega$ ) when there is no significant mixing of cell and pipette contents (depicted by icon). Arrow marks the time of establishing full access (fa;  $R_a = 4$  M $\Omega$ ) and the start of intracellular dialysis as depicted in the cell and patch-pipette icon which shows mixing of contents. A gradual decrease in firing rate was observed following intracellular dialysis with 100  $\mu$ M PKI<sub>5-24</sub>. A complete loss of firing occurred at 10 min. Lower traces show the spikes recorded at various time points. Note the truncated spikes in 1 due to lack of full access.

ing with patch electrodes containing control pipette solution (Fig. 3). However, on establishing whole-cell recording with patch electrodes containing pre-reduced CTX-A (1 or 5  $\mu$ g/ml) a  $235.3 \pm 79.1\%$  ( $n = 8$ ) increase in LC firing rates was observed within 2–10 min of attaining whole-cell access (Figs. 3 and 4). This is in contrast to the 50–110 min lag period observed with bath applications of the holotoxin (Fig. 1). Inclusion of PKI<sub>5-24</sub> in the patch pipette along with the CTX-A blocked the excitatory actions of CTX-A (Fig. 3) and completely inhibited firing in all the cells tested ( $n = 5$ ).

Intracellular applications of either the reduced or non-reduced forms of the B-subunit (1 or 10  $\mu$ g/ml) did not produce any excitation of LC neurones (Fig. 4); in fact there was a complete stoppage of firing in all the cells tested ( $n = 6$ ). Inclusion of NAD (Fig. 3) and/or DTT (not shown) alone in the patch-pipettes also failed to increase LC firing rates.

This is the first study that characterizes the electrophysiological actions of both extracellularly and intracellularly applied cholera toxin in CNS neurones, corroborating previously reported biochemical characterizations of cholera toxin action in various tissues. We show that bath application of cholera toxin causes a gradual and persistent increase in LC firing rates, with a characteris-

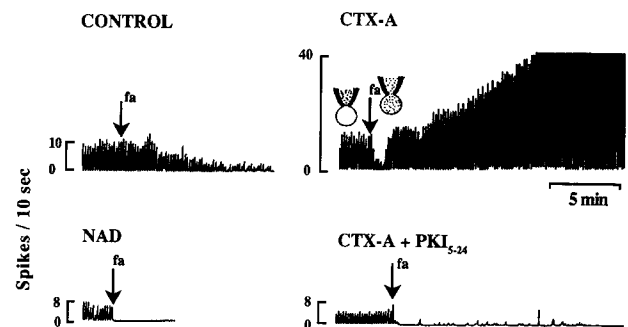


Fig. 3. Whole-cell recordings from 4 LC neurones demonstrating the effect on basal firing rates of intracellular dialysis with CTX-A and various other solutions. The effect of PKI<sub>5-24</sub> on CTX-A action is also shown. CONTROL: a gradual loss of spontaneous firing activity occurs following intracellular dialysis of an LC neurone with control patch-pipette solution. Arrow denotes the time at which full whole-cell access (fa) was established ( $R_a = 6$ –8 M $\Omega$ ). CTX-A: in contrast to the control, intracellular dialysis with a patch electrode containing pre-reduced CTX-A (1  $\mu$ g/ml) and NAD (100  $\mu$ M) produced an increase in LC firing rate. The cell had a basal firing rate of 1.6 Hz. A maximal rate of 11.3 Hz (out of recording range) was recorded 30 min after establishing whole cell recording and the cell stayed activated for the duration of the recording period (75 min). NAD: inclusion of NAD alone in the patch-pipette solution did not produce any excitation. Instead a loss of firing similar to that observed in a large proportion of LC neurones dialyzed with control pipette solution was seen. CTX-A + PKI<sub>5-24</sub>: addition of 100  $\mu$ M PKI<sub>5-24</sub> to a patch-pipette containing pre-reduced CTX-A prevented the increase in LC firing that is normally observed following CTX-A treatment.

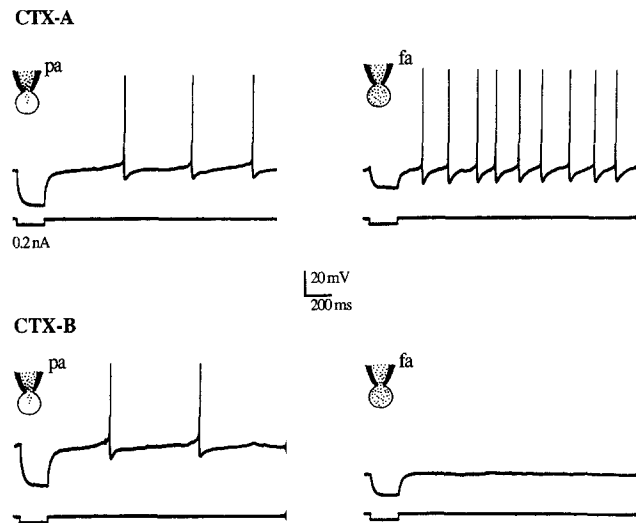


Fig. 4. Whole-cell recordings from two LC neurones showing the effect of intracellular dialysis with pipette solution containing CTX-A or CTX-B on spontaneous spike activity. CTX-A: left panel shows the 3 spikes occurring during an oscilloscope sweep recorded after incomplete rupture of the membrane patch ( $R_a = 10 \text{ M}\Omega$ ), i.e. before significant intracellular dialysis with CTX-A ( $1 \mu\text{g/ml}$ ) had occurred. Note the relatively high input impedance. No compensation was made for the high series resistance. Right panel shows the increased number of spikes recorded 12 min after establishing full whole-cell access ( $R_a = 4 \text{ M}\Omega$ ). CTX-B: left panel shows spikes recorded before significant intracellular dialysis with CTX-B ( $1 \mu\text{g/ml}$ ). A loss of firing similar to that seen with control pipette solution occurred on establishing whole-cell recording.

tic lag period of 50–110 min. The persistence of the response and the duration of the lag period correlate well with the observations reported in biochemical studies using various intact cell preparations [6, 7]. A lag period has also been observed in the blockade by cholera toxin of the opioid excitation in dorsal root ganglion explants [20]. The lag period has been attributed to the translocation of the active moiety of cholera toxin across the cell membrane [6, 7, 14]. The observed increases in LC firing rates following cholera toxin treatment were reversed by intracellular PKI<sub>5-24</sub>, a specific inhibitor of cAMP-dependent protein kinase. In light of these findings and our earlier demonstration that pacemaker activity in LC neurones is dependent on the cAMP-dependent phosphorylation pathway [2], we conclude that the excitatory actions of cholera toxin can be attributed to its  $G_s$ -mediated adenylate cyclase stimulating effect, which would cause an increase in intracellular cAMP levels. This in turn would increase LC pacemaker activity via the phosphorylation pathway.

In addition, we report that intracellular application of the A-subunit of cholera toxin via low-resistance patch electrodes mimics the excitatory actions of extracellularly applied holotoxin on LC firing rates, but without

the characteristic lag period observed with the holotoxin. In contrast to the excitatory actions of the A-subunit, intracellular applications of the B-subunit did not enhance LC firing rates; this is consonant with the reported lack of adenylate cyclase stimulating activity of the B-subunit [13]. These findings are consistent with biochemical studies which have shown that the biological activity of cholera toxin resides entirely in the A-subunit and that in membrane preparations [10, 13] the A-subunit stimulates adenylate cyclase activity without a significant lag period. PKI<sub>5-24</sub> blocked the excitation of LC neurones caused by intracellularly applied CTX-A. This parallels its effect on LC excitation induced by bath applications of the holotoxin.

To conclude, this study demonstrates that cholera toxin (A-subunit), which is known to produce persistent activation of the G protein  $G_s$ , increases firing rates in LC neurones via the cAMP-dependent phosphorylation pathway. An implication of this finding is that the activation of adenylate cyclase by any neurotransmitter receptor that is coupled to  $G_s$  would be expected to enhance the spontaneous firing rates of LC neurones. In fact, both vasoactive intestinal peptide [24] and corticotropin releasing factor [22], peptide transmitters that are coupled to  $G_s$ , are known to increase LC firing. Since the LC has the most extensive efferent network of any nucleus in the CNS, a transmitter-induced change in its basal activity via cAMP would modulate a variety of functions, such as the 'stress response' which is initiated by the release of corticotropin releasing factor.

We thank Ms. N. Margiotta for technical assistance and Ms. L. Fields for aiding in manuscript preparation.

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