Scorpion venom (*Leiurus quinquestriatus*) elicits accumulations of inositol phosphates and cyclic AMP in guinea pig cortical synaptoneurosomes

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Received 6 February 1986

Scorpion (*Leiurus quinquestriatus*) venom (ScV) stimulated accumulations of cyclic AMP and turnover of phosphatidylinositol in guinea pig cortical synaptoneurosomes. The concentrations of ScV that were necessary to increase cyclic [^{3}H]AMP accumulation were lower than those required to stimulate formation of [^{3}H]inositol phosphates from phosphatidylinositol. In the presence of 10 μM 2-chloroadenosine, ScV induced a dose-dependent synergistic accumulation of cyclic AMP with an EC_{50} value that was comparable to the EC_{50} required for stimulation of phosphatidylinositol turnover. Tetrodotoxin partially inhibited cyclic AMP accumulations elicited by ScV indicating that at least part of such responses are due to activation of voltage-dependent sodium channel. Tetrodotoxin virtually completely blocked formation of inositol phosphate stimulated by ScV. High concentrations of Mg^{2+} (30 mM) did not block responses to ScV indicating that release of neurotransmitters was not involved. Membrane potential changes could not be detected at concentrations of ScV that triggered the biochemical responses. Stimulation of phosphatidylinositol turnover by ScV appears to depend on an increase in influx of Na^+ in synaptoneurosomes, presumably due to slowing of the inactivation of voltage-dependent sodium channels by α-scorpion toxin, a component of ScV. At least in part, the stimulation of cyclic AMP accumulation by ScV correlates with increases in phosphatidylinositol turnover.

Na^+ channel *Tetrodotoxin* *cyclic AMP* *Phosphatidylinositol* *(Brain)* *Inositol phosphate*

1. INTRODUCTION

A variety of neurotransmitters and neuromodulators stimulate accumulations of cyclic AMP in brain preparations through interaction with specific receptors. Both direct activation of adenylate cyclase through the N_s-guanine nucleotide-binding protein and indirect pathways occur ([1] and reference therein). α_1-Adrenergic and H_1-histaminergic receptors appear to function primarily to augment accumulations of cyclic AMP elicited by activation of β-adrenergic, A_2-adenosine, VIP and H_2-histamine receptors in brain preparations. α_1-Adrenergic and H_1-histamine receptors also increase turnover of phosphatidylinositol in brain preparations as measured by accumulation of inositol phosphates [2]. It has been proposed that the breakdown of phosphatidylinositol by α_1-adrenergic, H_1-histaminergic, and serotonergic agonists and resultant generation of diacylglycerols are responsible for enhanced accumulations of cyclic AMP seen with such receptor agonists in brain preparations [3,4].

Recently, it was demonstrated that veratridine, batrachotoxin and other agents active at voltage-dependent sodium channels cause phosphatidylinositol breakdown in brain preparations [5]. Veratridine and batrachotoxin had previously been shown to cause accumulations of cyclic AMP in brain slices, but depolarization-elicited formation of adenosine and resultant stimulation of adenosine receptors were proposed to be primarily

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00145793/86/$3.50

responsible for the accumulations of cyclic AMP [5]. However, other factors were involved since sodium channel agents even in the presence of adenosine deaminase still elicit accumulations of cyclic AMP [7] and augment responses of cyclic AMP elicited by 2-chloroadenosine or forskolin [8]. It was suggested that effects of sodium channel agents on cyclic AMP accumulations in part might be mediated by increases in phosphatidylinositol turnover [8]. An α-scorpion toxin, primarily responsible for biological activity of scorpion venom (ScV) of *Leiurus quinquestriatus*, slows inactivation of sodium channels without inducing marked depolarization of nerve or muscle [9]. The effects of ScV on cyclic AMP accumulation and breakdown of phosphatidylinositol turnover in a guinea pig cerebral cortical synaptoneurosome preparation have now been compared to provide insights into possible correlations of these two responses. The synaptoneurosome preparation contains many synaptosomes with attached resealed post-synaptic entities (neurosomes) [10] and is proving a useful, readily obtained, brain preparation for investigation of cyclic AMP generation ([10] and references therein), phosphatidylinositol turnover [4,5] and ion fluxes [11].

2. MATERIALS AND METHODS

2.1. *Materials*

Scorpion venom (*L. quinquestriatus*), tetrodotoxin (TTX), adenosine deaminase and 2-chloroadenosine were from Sigma (St. Louis, MO). [^{3}H]Inositol (spec. act. 14 Ci/mmol) was from Amersham (Arlington Heights, IL). [^{3}H]-Adenine (spec. act. 12.8 Ci/mmol) was from New England Nuclear (Boston, MA). [^{3}H]Triphenylmethylphosphonium bromide (spec. act. 63 mCi/mmol) was prepared as described [12]. Hydrofluor and Betafluor were purchased from National Diagnostics (Somerville, NJ).

2.2. *Synaptoneurosomes*

Male Hartley guinea pigs (175–220 g) were decapitated and the brains rapidly removed. The cerebral cortex was dissected out and synaptoneurosomes obtained according to Hollingsworth et al. [10]. Briefly, the cortex of one brain was homogenized in 7–10 vols Krebs-Henseleit buffer in a glass-glass homogenizer (5 strokes). The suspension was centrifuged at 1000 × g for 10 min, the supernatant decanted and the pellet reconstituted in an appropriate volume of buffer. The composition of Krebs-Henseleit buffer was as follows: NaCl, 118.5 mM; KCl, 4.7 mM; $MgSO_4$, 1.18 mM; $CaCl_2$, 2.5 mM; KH_2PO_4, 1.18 mM; $NaHCO_3$, 24.9 mM; glucose, 10 mM. The pH was maintained at 7.4 by continuous gassing of O_2:CO_2 (9:5).

2.3. *Cyclic AMP accumulations*

The pellet from one brain was resuspended in 20 ml buffer and incubated under O_2:CO_2 (95:5) with 0.6 μM [^{3}H]adenine and 5 units/ml of adenosine deaminase for 45–60 min at 37°C, followed by washing twice with fresh buffer and resuspension in 35–40 ml buffer. The suspension of adenine-labelled synaptoneurosomes was transferred in 1-ml aliquots to scintillation vials and allowed to incubate for 10 min at 37°C. Agents or buffer were added in 100 μl, followed by a 10 min incubation. The contents of the vials were transferred to microfuge tubes, which were centrifuged for 60 s in a microcentrifuge. The supernatant was decanted and the pellet resuspended in 1.2 ml of 6% trichloroacetic acid containing 1 mM cyclic AMP. The percent conversion of radioactive adenine nucleotides to cyclic [^{3}H]AMP was determined as described [13].

2.4. *Phosphatidylinositol turnover*

The pellet from one brain was resuspended in 20 ml buffer with 1 μM [^{3}H]inositol and incubated for 60 min at 37°C with gentle bubbling of O_2:CO_2 (95:5). The [^{3}H]inositol-labelled synaptoneurosomes were collected by centrifugation and resuspended in 12–15 ml buffer containing 10 mM LiCl. The suspension was distributed in polypropylene tubes (1 mg protein/tube). Agents were added in 20 μl. The tubes were gassed briefly with O_2:CO_2, capped and incubated for 90 min at 37°C. The tubes were centrifuged, the pellet washed with buffer to remove the free [^{3}H]inositol, and 1 ml of 6% trichloroacetic acid added. The tubes were vortex-mixed and then centrifuged. [^{3}H]Inositol phosphates were analyzed in the supernatant as described [14]. In addition, the trichloroacetic acid precipitate was resuspended in 0.5 ml of a 1:1 mixture of 10 mM inositol in aqueous 1 M KCl and methanol and 0.5 ml

chloroform added. The tubes were mechanically shaken for 5 min and subsequently centrifuged at 1000 × *g* to separate the two phases. The aqueous phase was discarded and an aliquot of the lower organic phase dried and mixed with Betafluor for scintillation counting. The major radioactive compound in this extract was shown by thin-layer chromatographic analysis to be [^{3}H]phosphatidylinositol (not shown). The amount of [^{3}H]phosphatidylinositol present in each assay was used to normalize the amount of [^{3}H]inositol phosphates to that formed with control synaptoneurosomes.

2.5. *Membrane potentials*

The membrane potentials in synaptoneurosomes were measured with [^{3}H]triphenylmethylphosphonium ($TPMP^+$) uptake as described [12]. Briefly, to prewarmed tubes (37°C) containing 80 μM [^{3}H]$TPMP^+$ bromide in Krebs-Ringer glucose bicarbonate buffer, pH 7.4 (final volume 0.1 ml), 0.01 ml synaptoneurosome suspension was added. Incubations were terminated after 5 min by diluting the reaction mixture with 4 ml ice-cold phosphate-buffered normal saline (pH 7.4) and immediately filtering with vacuum through 0.5 μm EH Millipore filters. Values were expressed as percentage of $TPMP^+$ uptake of control synaptoneurosomes. The control synaptoneurosomes had a resting membrane potential of −78 mV as calculated from the ratio $[TPMP^+]_i:[TPMP^+]_o$ at equilibrium and the Nernst equation [14].

3. RESULTS

The ScV from *L. quinquestriatus* induced an accumulation of cyclic [^{3}H]AMP in a dose-dependent fashion in guinea pig cerebral cortical synaptoneurosomes (fig.1A). However, the dose-response curve was clearly biphasic. The high-affinity component had an EC_{50} of 0.3 μg/ml ScV. The α-scorpion toxin (M_r ~7000) has been reported to comprise 1% of ScV [9] and an EC_{50} of 0.03 μg/ml ScV would, therefore, correspond to a concentration of about 0.4 nM α-scorpion toxin.

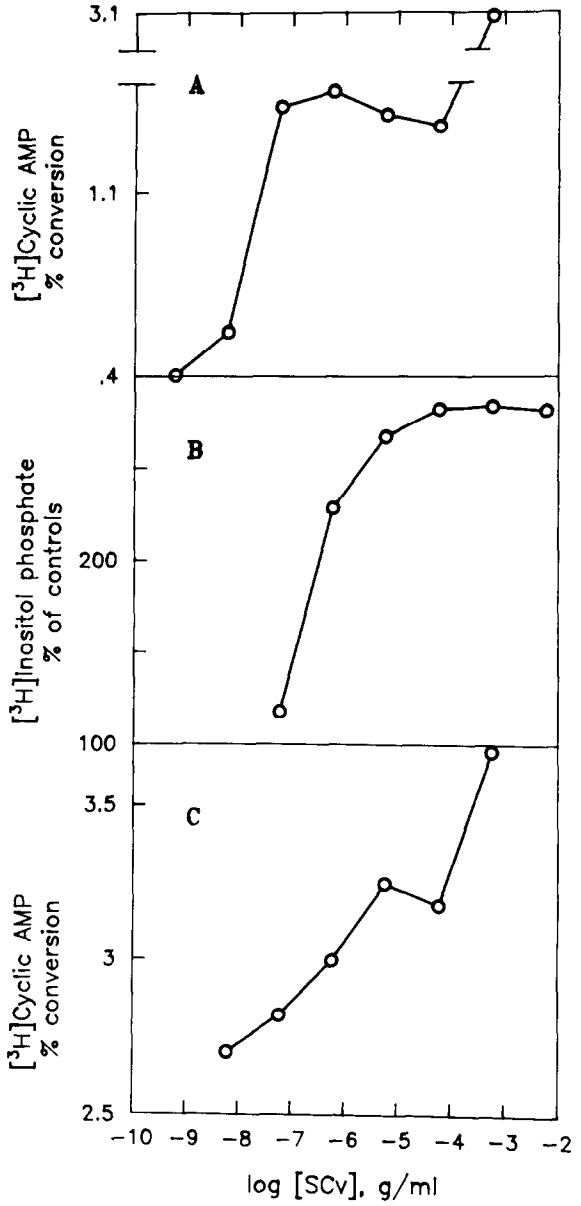

Fig.1. Effect of scorpion venom (ScV) on accumulation of cyclic [^{3}H]AMP (A,C) and formation of [^{3}H]inositol phosphate (B) in guinea pig cerebral cortical synaptoneurosomes. (A) Cyclic [^{3}H]AMP accumulation was determined in [^{3}H]adenine-labeled synaptoneurosomes after 10 min with varying concentrations of ScV, as described in section 2. Values are means of 2 experiments. (B) [^{3}H]Inositol phosphate formation was determined in [^{3}H]inositol-labeled synaptoneurosomes after 90 min with varying concentrations of ScV and 10 mM LiCl as described in section 2. Values are from a representative experiment. (C) Cyclic [^{3}H]AMP accumulation in [^{3}H]adenine-labeled synaptoneurosomes was determined after 10 min presence of 10 μM 2-chloroadenosine and varying concentrations of ScV described in section 2. Values are means of 2 experiments.

At higher concentrations of ScV further increases in cyclic AMP accumulations occurred (fig.1A). The ScV caused a dose-dependent increase in formation of [^{3}H]inositol phosphates in guinea pig cerebral cortical synaptoneurosomes (fig.1B). In this case, the dose-response curve was monophasic with an EC_{50} of 0.3 μg/ml ScV. This EC_{50} is greater by an order of magnitude than the high-affinity EC_{50} for ScV-elicited accumulation of cyclic AMP. Indeed at 60 μg/ml ScV, the high-affinity component of cyclic AMP accumulation is already maximal, while stimulation of formation of inositol phosphates by ScV is barely detectable. ScV markedly enhanced accumulations of cyclic AMP elicited by 10 μM 2-chloroadenosine (fig.1C). The increase in cyclic AMP accumulation over that in the presence of 2-chloroadenosine alone reached a maximum at a dose of ScV of 10–100 μg/ml. The EC_{50} of ScV was about 0.5 μg/ml, much higher than that for ScV-elicited accumulation of cyclic AMP in the absence of 2-chloroadenosine (fig.1A) and similar in magnitude to the EC_{50} for ScV-elicited formation of inositol phosphates.

The inhibition by tetrodotoxin of ScV-elicited cyclic AMP accumulation and formation of inositol phosphates was dose-dependent (fig.2A). But maximal inhibition of cyclic AMP accumulation by TTX of the ScV-elicited accumulation of cyclic AMP was only 50–60% even at 10 μM TTX. TTX inhibited ScV-elicited stimulation of phosphatidylinositol turnover with an IC_{50} of 100–200 nM (fig.1B). In this case the higher doses of TTX inhibited the ScV response nearly completely (80–90%).

To ascertain whether the responses to ScV were due to ScV-evoked release of a neurotransmitter, the effects of the presence of high concentrations of Mg^{2+} (30 mM) on responses to ScV were determined. Such concentrations of magnesium are often used to antagonize calcium-dependent release of neurotransmitters. The effects of ScV were not blocked by magnesium (table 1). However, high magnesium alone caused an increase in both cyclic AMP accumulations and formation of inositol phosphates.

ScV did not cause changes in membrane potential (table 2) at concentrations that were effective in producing the biochemical responses. At high concentrations ScV did cause depolarization of

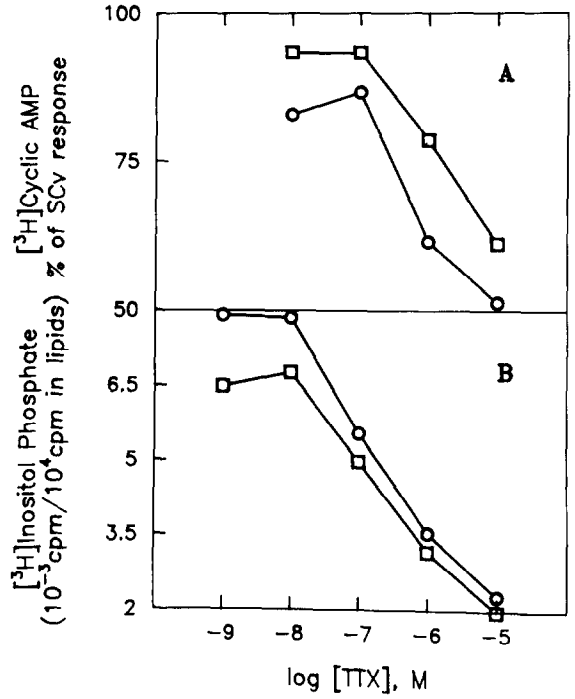

Fig.2. Effect of tetrodotoxin (TTX) of scorpion venom (ScV)-elicited accumulation of cyclic [^{3}H]AMP (A) and formation of [^{3}H]inositol phosphate (D) in guinea pig cerebral cortex synaptoneurosomes. Synaptoneurosomes were incubated with 6 μg/ml (○) or 60 μg/ml (□) ScV in the presence of various concentrations of tetrodotoxin and either cyclic [^{3}H]AMP (A) or [^{3}H]inositol phosphate (B) was determined as described in section 2. Values are means from 2 experiments (A) or are from a representative experiment, each point in triplicate.

synaptoneurosomes as measured by reductions in uptake of [^{3}H]TPMP$^+$.

4. DISCUSSION

Depolarizing agents, such as veratridine and batrachotoxin, are well known to cause accumulations of cyclic AMP in brain preparations [6] and recently have been demonstrated to cause phosphatidylinositol breakdown [5]. Breakdown of phosphatidylinositol results in formation of two 'second messengers', namely inositol phosphates, certain of which can increase release of intracellular Ca^{2+}, and diacylglycerols, which, in consort with Ca^{2+}, can activate the enzyme protein kinase C. A variety of compounds active at

Table 1

Effect of ScV and/or Mg^{2+} on accumulation of cyclic [^{3}H]AMP or formation of [^{3}H]inositol phosphate in guinea pig cerebral cortical synaptoneurosomes

Agent	Cyclic [^{3}H]AMP (% conversion)[a]	[^{3}H]Inositol phosphates (% of controls)[b]
Control	0.47 ± 0.08	100
Mg^{2+} (30 mM)	0.99 ± 0.20	169
ScV (6 μg/ml)	1.80 ± 0.08	214
ScV + Mg^{2+}	1.65 ± 0.30	297

[a] Synaptoneurosomes, labeled with [^{3}H]adenine, were incubated with agents for 10 min and accumulation of cyclic [^{3}H]AMP was determined as described in section 2. Values are means ± SE for 3 determinations

[b] Synaptoneurosomes, labeled with [^{3}H]inositol, were incubated with agents and 10 mM LiCl for 90 min and formation of [^{3}H]inositol phosphate was determined as described in section 2

voltage-dependent sodium channels including not only veratridine and batrachotoxin, but also aconitine, various scorpion toxins, pumiliotoxin B and pyrethroids, cause breakdown of phosphatidylinositol [5]. Other agents that would induce an increase in intracellular sodium, such as ouabain, monensin and high concentrations of K^+, also induced turnover of phosphatidylinositol, suggesting that not the depolarization, but rather the increase in intracellular Na^+ was responsible for enhancing phosphatidylinositol turnover. In view of the recent observations linking activation of protein kinase C with enhanced responsiveness of cyclic AMP-generating systems in brain [3], pinealocytes [15], pheochromocytoma cells [16] and other cells, it appeared likely that agents that increase intracellular sodium and thereby increase phosphatidylinositol breakdown should augment cyclic AMP accumulations. Indeed, ScV, which contains the sodium channel agent α-scorpion toxin, even in the presence of adenosine deaminase, does increase cyclic AMP levels in guinea pig synaptoneurosomes (fig.1A). ScV also enhances 2-chloroadenosine-elicited accumulations of cyclic AMP (fig.1C) and stimulates formation of inositol phosphates (fig.1B).

Table 2

Effect of ScV on uptake of [^{3}H]$TPMP^+$ in guinea pig cerebral cortical synaptoneurosomes

[ScV] (μM)	$TPMP^+$ uptake (% of controls)
0.6	118
3	96, 87
6	73 ± 4
15	49, 52
30	49 ± 5
60	57, 43
120	58

Synaptoneurosomes were incubated with [^{3}H]$TPMP^+$ and various concentrations of ScV for 5 min and uptake determined. Values are means ± SE for 3 determinations or are individual values

Receptor-mediated stimulation of phosphatidylinositol breakdown has been proposed to be linked to enhanced responsiveness of cyclic AMP-generating systems seen with α_1-adrenergic, H_1-histamine and serotonin receptor agonists in brain slices [3] and with α_1-adrenergic receptors in pinealocytes [15]. However, muscarinic agonists increase phosphatidylinositol breakdown in brain slices without augmenting cyclic AMP accumulations [4]. The present results indicate that sodium channel agents, such as those present in ScV, may also enhance cyclic AMP accumulations through stimulation of phosphatidylinositol breakdown. However, ScV markedly stimulates cyclic AMP accumulations at concentrations that have minimal effects on phosphatidylinositol breakdown (cf. fig.1A,B). Such results indicate that either: (i) the effects of the enhanced influx of Na^+ elicited by ScV on cyclic AMP accumulation and on phosphatidylinositol breakdown are independent phenomena; or (ii) in a heterogeneous system, such as that of brain synaptoneurosomes, cyclic AMP accumulations occur in compartments in which phosphatidylinositol turnover is more sensitive to the effects of ScV than in other major compartments. It should be noted that there is a relatively good correlation between the potency of ScV in eliciting phosphatidylinositol breakdown and that in enhancing cyclic AMP accumulation in the presence of 2-chloroadenosine (cf. fig.1B,C).

The role of voltage-dependent sodium channels in the responses to ScV in guinea pig synaptoneurosomes was assessed with tetrodotoxin.

Tetrodotoxin completely blocked ScV-elicited formation of inositol phosphate (fig.2B), indicating that this response involved the function of voltage-dependent sodium channels. However, the accumulation of cyclic AMP elicited by ScV was only partially blocked by tetrodotoxin (fig.2A), suggesting that mechanisms not involving sodium channels or phosphatidylinositol turnover account for part of the stimulation of cyclic AMP-generating systems by ScV in guinea pig synaptoneurosomes.

Elevated concentrations of extracellular Mg^{2+}, which were used for the purpose of blocking possible ScV-elicited calcium dependent release of neurotransmitter, did not block either ScV-elicited cyclic AMP accumulation or phosphatidylinositol breakdown (table 1). Indeed, magnesium alone caused both cyclic AMP accumulation and phosphatidylinositol breakdown. Stimulatory effects of magnesium on cyclic AMP accumulations in brain slices have been noted [17]. The results suggest that magnesium may enhance cyclic AMP accumulations in brain preparations through activation of phosphatidylinositol breakdown. The mechanism involved is unclear, but may be related to effects of elevation of internal sodium on cyclic AMP and phosphatidylinositol turnover. Thus, activation of the Na^+/Mg^{2+} antiport [18], by elevated internal levels of sodium could enhance magnesium influx. Similarly, elevation of internal levels of sodium could enhance calcium influx through activation of a Na^+/Ca^{2+} antiport. The calcium ionophore ionomycin, which would elevate calcium influx in a nonspecific manner, does augment cyclic AMP accumulations and stimulates formation of inositol phosphates in guinea pig synaptoneurosomes [8]. Calcium and/or magnesium act as cofactors for enzymes (adenylate cyclase, phospholipase C) involved in generation of second messengers, whose formation is enhanced by sodium-channel agents and by elevated extracellular concentrations of magnesium. However, while calcium is stimulatory to phospholipase C and related phospholipases, magnesium has been found to be inhibitory [19]. Further studies are required to delineate the mechanisms whereby activation of sodium channels or the presence of high concentrations of extracellular magnesium can invoke an increased rate of phosphatidylinositol breakdown, and to define further the significance of phosphatidylinositol breakdown to enhanced responses of cyclic AMP-generating systems in brain and other tissues.

ACKNOWLEDGEMENT

The authors wish to thank E. McNeal for assistance in measurement of membrane potential.

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