AN IN VITRO SYSTEM TO STUDY THE ACTION POTENTIAL SODIUM CHANNEL

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1. Introduction

The fast sodium channel is sensitive to a variety of toxins. Veratridine, batrachotoxin, sea anemone and scorpion neurotoxins act on the gating system of the Na^+ channel [1–6] and change its ionic selectivity [2,7], thereby provoking $^{86}Rb^+$ or $^{42}K^+$ efflux from preloaded excitable cells [8,9]. This stimulated $^{86}Rb^+$ (or $^{42}K^+$) efflux is blocked by tetrodotoxin. We have used this property to devise an in vitro assay with synaptosomes. $ScTX_{II}$ and ATX_{II} stimulate $^{86}Rb^+$ release through the Na^+ channel without changing the synaptosomal membrane potential. Moreover, toxin-induced release of $^{86}Rb^+$ requires the presence of Na^+ (or Li^+) in the external medium, suggesting that the formation of a conducting form of the Na^+ channel is dependent upon both the action of toxins and the saturation of external Na^+ sites.

2. Materials and methods

Rat brain synaptosomes were prepared according to [10] and used immediately after preparation. Scorpion toxin II was purified from *Androctonus australis* Hector according to [11]; sea anemone toxin II from *Anemonia sulcata* according to [12] with minor modifications. Veratridine and 3,4-diaminopyridine were purchased from Aldrich, tetraethylammonium from Merck, tetrodotoxin from Sankyo. Batrachotoxin was kindly given to us by Drs J. Daly and Tokuyama and $[^3H]TTP^+$ by Dr Kaback. $^{86}Rb^+$ efflux experiments and membrane potential measurements using $[^3H]TTP^+$ were performed as in the figure legends.

Abbreviations: ATX_{II}, sea anemone *(Anemonia sulcata)* toxin II; $ScTX_{II}$, scorpion *(Androctonus australis)* toxin II; TTX, tetrodotoxin; TEA, tetraethylammonium; TPP^+, tetraphenylphosphonium; 3,4-D, 3,4-diaminopyridine; BTX, batrachotoxin; Vera, veratridine

3. Results and discussion

Synaptosomes accumulate $^{86}Rb^+$ or $^{42}K^+$ in a Na^+-free medium. Maximal accumulation (~10-times) is reached after 20 min and ionic levels remain stable for at least an additional 10 min. The presence of 1 μM ATX_{II} or of 0.1 mM veratridine in the Na^+-free medium does not modify the time course of ^{86}Rb accumulation. The addition of 120 mM Na^+ to $^{86}Rb^+$-loaded synaptosomes preincubated with ATX_{II}, $ScTX_{II}$, veratridine or batrachotoxin provokes a rapid release of $^{86}Rb^+$ to a new equilibrium level. Stimulation of $^{86}Rb^+$ release is completely blocked by 1 μM TTX. Identical results were obtained using $^{42}K^+$-loaded synaptosomes. Toxin-activated $^{86}Rb^+$ (or $^{42}K^+$) efflux is not affected by specific blockers of the K^+ channel such as TEA and 3,4-diaminopyridine even when they are present at high concentrations (fig.1). These observations confirm that as for neuroblastoma cells, $^{86}Rb^+$ or $^{42}K^+$ efflux is through the Na^+ channel.

Dose–response curves for $^{86}Rb^+$ or $^{42}K^+$ release induced by the gating-system toxins are presented in fig.2. Experiments with veratridine show similar results for $^{42}K^+$- and $^{86}Rb^+$-loaded synaptosomes. Half-maximal effects are observed at 0.8 nM $ScTX_{II}$, 13 nM ATX_{II}, 1.4 μM batrachotoxin and 27 μM veratridine. True dissociation constants obtained for the toxin–receptor complexes using radiolabelled toxins were 0.6 nM for $ScTX_{II}$, a value very close to that found here, and 0.15 μM for ATX_{II}, which is an

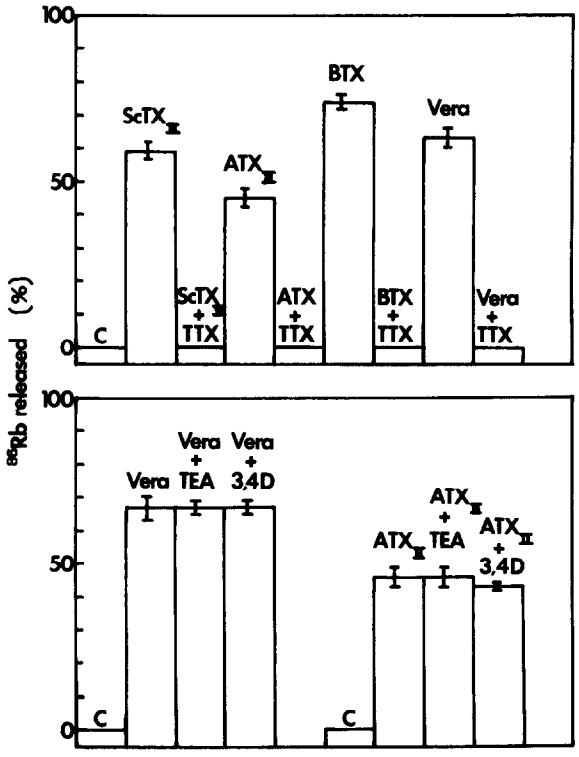

Fig.1. Influence of neurotoxins on $^{86}Rb^+$ efflux from preloaded synaptosomes. Synaptosomes (~2 mg/ml) were suspended in 400 μl of the following medium: 3.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 20 mM Tris–HCl at pH 7.4, 140 mM sucrose, [^{14}C]sucrose (Amersham, 1 μCi/ml), 40 mM choline chloride, 0.05% bovine serum albumin, tracer amounts of ^{86}RbCl (2–6 μCi/ml) or ^{42}KCl (4 μCi/ml) and the desired neurotoxins. After 20 min incubation at 21°C, $^{86}Rb^+$ release was triggered by adding 4 M NaCl to a final external [Na^+] of 120 mM. After an additional 10 min, synaptosomes were centrifuged for 3 min at 15 000 rev./min at room temperature. Pellet and supernatant were counted separately in 8 ml Picofluor 30 (Packard). Pellet radioactivity was corrected for $^{86}Rb^+$ in extravesicular space using [^{14}C]sucrose. Toxins used are: scorpion toxin II ($ScTX_{II}$) 0.1 μM; sea anemone toxin II (ATX_{II}) 10 μM; veratridine (Vera) 0.1 mM; batrachotoxin (BTX) 30 μM; tetrodotoxin (TTX) 1 μM; tetraethylammonium (TEA) 10 mM; 3,4-diaminopyridine (3,4-D) 0.2 mM; c (control) no toxin added.

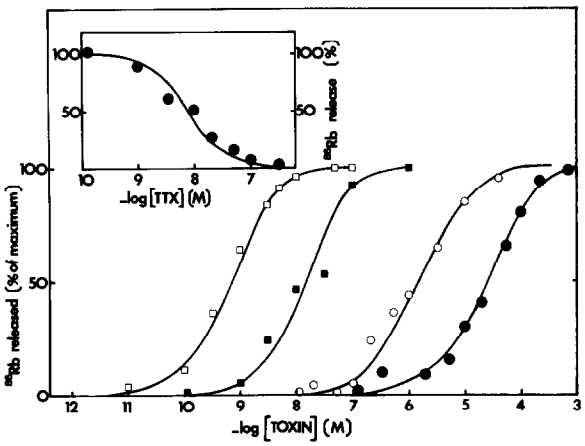

Fig.2. Dose–response curves of $^{86}Rb^+$ efflux stimulated by veratridine (•), batrachotoxin (○), ATX_{II} (▪), $ScTX_{II}$ (□). Inset: TTX inhibition of 0.5 μM ATX_{II}-induced $^{86}Rb^+$ release.

order of magnitude higher than the value we observed [13]. However, there are both high and low affinity binding sites for ATX_{II} [14], and it may be that direct binding studies have only identified the low affinity sites. Dose–response curves with veratridine and batrachotoxin (fig.2) are very similar to those found for toxin-stimulated $^{22}Na^+$ entry into neuroblastoma cells [14–16]. Inhibition of ATX_{II}-stimulated $^{86}Rb^+$ efflux by increasing concentrations of TTX is shown in the inset of fig.2. Half-maximal inhibition is observed at 3.9 nM. This value is very close to the true dissociation constant (5 nM) of the TTX–receptor complex obtained using synaptosomes under the same experimental conditions.

Membrane potentials have been measured with the radiolabelled permeant lipophilic cation, [^{3}H]tetraphenylphosphonium (TPP^+), which gives potential values very similar to those determined with intracellular microelectrodes [17]. TTP^+ was recently used with success to measure perturbations of the synaptosomal membrane potential by *Tetanus* toxin [18]. The influence of veratridine, batrachotoxin, $ScTX_{II}$ and ATX_{II} on synaptosomal membrane potential is presented in fig.3. At high concentrations, batrachotoxin and veratridine almost completely depolarize the synaptosomal membrane; this effect is totally blocked by TTX. Dose–response curves for the stimulation of $^{86}Rb^+$ release by veratridine and batrachotoxin are very similar to those in fig.3. $ScTX_{II}$ and ATX_{II} do not produce variations in potential even at the highest concentrations tested. These results are consistent with the known mechanism of action of the different toxins. Veratridine and batrachotoxin permanently activate the fast Na^+ conductance with the resultant depolarization of excitable cells [1–4]; this activation is blocked by TTX. In contrast, ATX_{II} and $ScTX_{II}$ selectively retard the inactivation of the Na^+ channel [5,6] and thereby do not depolarize

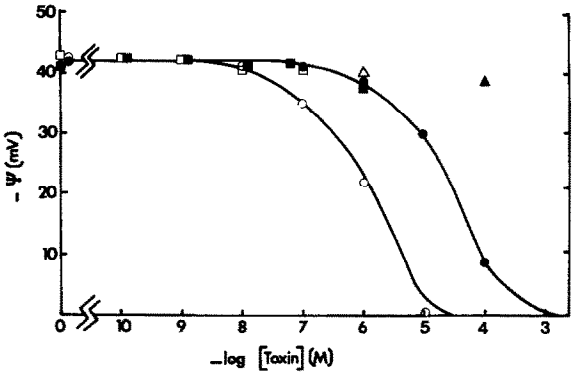

Fig.3. Influence of neurotoxins on synaptosomal membrane potential. Synaptosomes (2 mg/ml) were incubated at 37°C for 10 min in the following medium: 140 mM NaCl, 5 mM KCl, 2.8 mM $CaCl_2$, 1.3 mM $MgSO_4$, 20 mM Tris–HCl at pH 7.4 10 mM glucose, 10 μCi/ml [^{3}H]TPP$^+$ in the presence of varied concentrations of neurotoxins. The amount of [^{3}H]TPP$^+$ was determined after filtration on EHWP filters. Free [TPP$^+$] was determined from radioactivity in the supernatant after centrifugation of an aliquot of the incubation medium at 15 000 rev./min for 1 min. Zero potential value was determined after incubation of synaptosomes in the same medium containing 15 mM NaCl and 200 mM KCl. Toxin used were $ScTX_{II}$ (□), ATX_{II} (■), veratridine (●), veratridine plus 1 μM TTX (▲), batrachotoxin (○) and batrachotoxin plus 1 μM TTX (△).

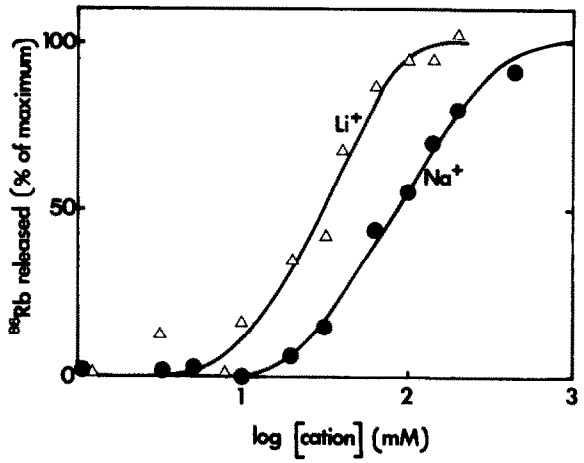

Fig.4. Dose–response curve for Na$^+$ (●)- and Li$^+$ (△)-stimulated ^{86}Rb$^+$ efflux triggered by 1 μM ATX_{II}. Experimental conditions are the same as those in fig.1, except that [Na$^+$] and [Li$^+$] were varied and cholinium$^+$ added so that [cholinium$^+$] plus [Na$^+$] or [Li$^+$] was 200 mM. Under these conditions the highest [Na$^+$] or [Li$^+$] used did not depolarize the synaptosomes. Curve fitting was performed according to [9].

excitable membranes at the low concentration used here (fig.3 and J. Daly, personal communication). The fact that ATX_{II} and $ScTX_{II}$ stimulate $^{86}Rb^+$ release from synaptosomes without depolarizing the membrane is another indication that $^{86}Rb^+$ is not released through the K^+ channel which is activated by membrane depolarization. However, the most important conclusion is that the Na^+ channel can be studied in vitro at constant membrane potential.

Stimulation of $^{86}Rb^+$ (or $^{42}K^+$) release by the 4 toxins used here is not observed when the Na^+ in the external medium is replaced by cholinium$^+$, although it is observed when Na^+ is replaced by other permeant cations such as Li^+. Fig.4 shows the dose–response effects of Na^+ and Li^+ on $^{86}Rb^+$ release induced by ATX_{II}. Half-maximal effects are observed at 85 mM Na^+ and 31 mM Li^+; positive cooperativity was observed, with Hill coefficients of 1.5 and 1.7 for Na^+ and Li^+, respectively. This result is interesting in two different respects.

(1) It suggests that there are multiple sites for Na^+ (or Li^+) at the external face of the Na^+ channel structure, and that these sites (probably at least two with a Hill coefficient of 1.5) are in homotropic interaction. This observation confirms results obtained with neuroblastoma cells [14,19].

(2) The toxins, which are known to activate the Na^+ channel, are unable by themselves to stabilize a conducting form of this structure. A conducting form of the Na^+ channel requires both the association of the toxin with its own site and the saturation of external Na^+ sites. In other words, external Na^+ sites of the Na^+ channel are involved in the control of the gating system.

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