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Effect of tunicamycin on the expression of functional brain neurotransmitter receptors and voltage-operated channels in *Xenopus* oocytes

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The role of *N*-glycosylation on the expression of functional brain neurotransmitter receptors and voltage-operated channels was studied by injecting *Xenopus* oocytes with mRNA from rat brain or chick optic lobe, and culturing them in the presence or absence of tunicamycin, an inhibitor of asparagine linked glycosylation. Electrophysiological recordings were then made to assess the amounts of functional receptors and channels present in the oocyte membrane. The appearance of γ -aminobutyric acid (GABA) receptors and voltage-activated Na^+ channels was profoundly reduced. In contrast, the functional expression of kainate receptors, and voltage-activated K^+ and Ca^{2+} channels was much less affected. Thus, it seems that kainate receptors, and K^+ and Ca^{2+} channels can be expressed and function normally without being glycosylated. On the other hand, GABA receptors and Na^+ channels may need to be *N*-glycosylated in order to function properly, or to ensure their correct insertion into the membrane.

INTRODUCTION

It is known that some neurotransmitter receptors (e.g. glycine, kainate, γ -aminobutyric acid (GABA) and nicotinic and muscarinic acetylcholine receptors) and voltage-operated channels (Na^+ and Ca^{2+} channels) in the central nervous system bind to immobilized lectins, indicating that these molecules are glycoproteins^{5,13,14,21,30,33,34,37,41}. Furthermore, in brain glycoproteins about 90% of the carbohydrate moieties are attached to asparagine residues¹⁶. Thus, it appears that many receptor and channel proteins in the brain are *N*-glycosylated. In the cases of nicotinic acetylcholine receptors (AChR) in cultured muscle cells, and Na^+ channels in cultured muscle or neuronal cells, it was shown, by using tunicamycin to block protein glycosylation, that *N*-linked carbohy-

drate attachment is required for functional expression in the cell surface membrane^{2,4,20,31,32,40}. However, with the exception of the Na^+ channels³², it is not yet clear whether the *N*-linked carbohydrate moieties found on other membrane channels, and receptors, in the brain are essential for their function.

To a large extent this has been due to difficulties in studying the ways in which these molecules are synthesized in the brain. An alternative approach has recently become available, with the finding that *Xenopus* oocytes, injected with brain mRNA, are able to express many neurotransmitter receptors and voltage-operated channels^{6,9,17,23,24,27,36}. Here we have used this system to investigate the role of *N*-glycosylation in the expression of functional brain receptors and channels using tunicamycin, an inhibitor of asparagine linked glycosylation³⁹.

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MATERIALS AND METHODS

The experiments were carried out from 1982 to 1985 at University College London and U.C. Irvine, using *Xenopus laevis* oocytes, which were injected with poly(A)⁺ mRNA derived from the optic lobe of 20-day-old chick embryos, or from the brain of adult Wistar rats. Methods for isolation and injection of mRNA, for collagenase treatment of oocytes to remove enveloping cells, and for electrophysiological recording were as described previously^{6,17,23,24,27}. Oocytes obtained from each single donor were divided into 3 batches, which were injected with the same amount of mRNA, and were examined 3–5 days after injection, unless stated otherwise. The first batch (control) was maintained in normal Barths solution after injection. The second batch was incubated in Barths solution containing tunicamycin (2 µg/ml) for one day before injection to deplete the oocytes of glycosylation intermediates, and then continuously until used for recording. The third batch was similarly treated with external tunicamycin, but was additionally injected with 0.2 ng tunicamycin at a concentration of 40 µg/ml⁻¹, which was added to the mRNA solution for injection. During electrophysiological recording all oocytes were perfused with normal frog Ringer solution (without tunicamycin), at 19–21 °C.

RESULTS

Tunicamycin does not inhibit protein synthesis

Tunicamycin is widely used as an inhibitor of *N*-glycosylation³⁹, but we were first concerned to exclude the possibility that it also inhibited protein synthesis. For this purpose, mRNA-injected oocytes were assayed for incorporation of [³⁵S]methionine into newly synthesised proteins, measured following trichloroacetic acid (TCA) precipitation on filters³.

The TCA precipitable radioactivity was measured in paired batches (10 oocytes in each) of control oocytes and oocytes treated externally with 2 µg/ml tunicamycin. [³⁵S]Methionine was added shortly after injection of mRNA, and measurements were made two days later. The mean counts obtained from 4 experiments were; control = $4.1 \times 10^6 \pm 0.2 \times 10^6$ cpm/oocyte (S.E.M.), tunicamycin-treated = $4.2 \times 10^6 \pm 0.3 \times 10^6$ cpm/oocyte. Thus, tunicamycin does not appear to inhibit the translation of mRNA under the

conditions used in the present experiments.

Effect of tunicamycin on voltage-activated channels induced by chick optic lobe mRNA

Depolarization of oocytes injected with poly(A)⁺ mRNA derived from chick optic lobe (c.o.l. mRNA) elicits two main currents, which are not usually present in native (non-injected) oocytes^{29,35}. The first is a fast inward current, which becomes maximal at a potential of about -10 mV, is blocked by tetrodotoxin (TTX) and is carried largely by sodium ions (Fig. 1A; see also ref. 7). The second is a slower outward current which continues to increase with increasing depolarization and is carried largely by potassium ions⁷. In addition to these currents, depolarization to around 0 mV also elicits a slower transient outward (T_{out}) current, which is carried by chloride ions and depends upon an influx of calcium which activates endogenous calcium-dependent chloride membrane channels^{1,22,25,27}. The T_{out} current is often present in native oocytes, but is enhanced after injection of brain mRNA^{8,35}, probably because the mRNA in-

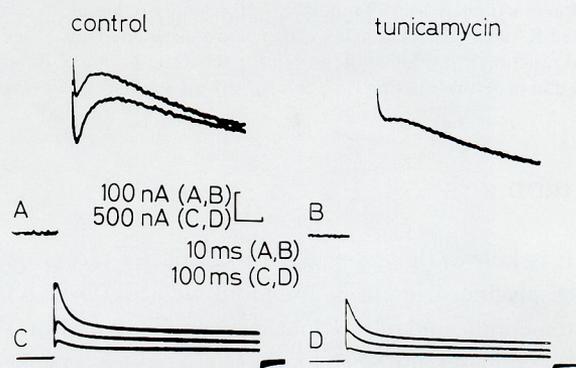
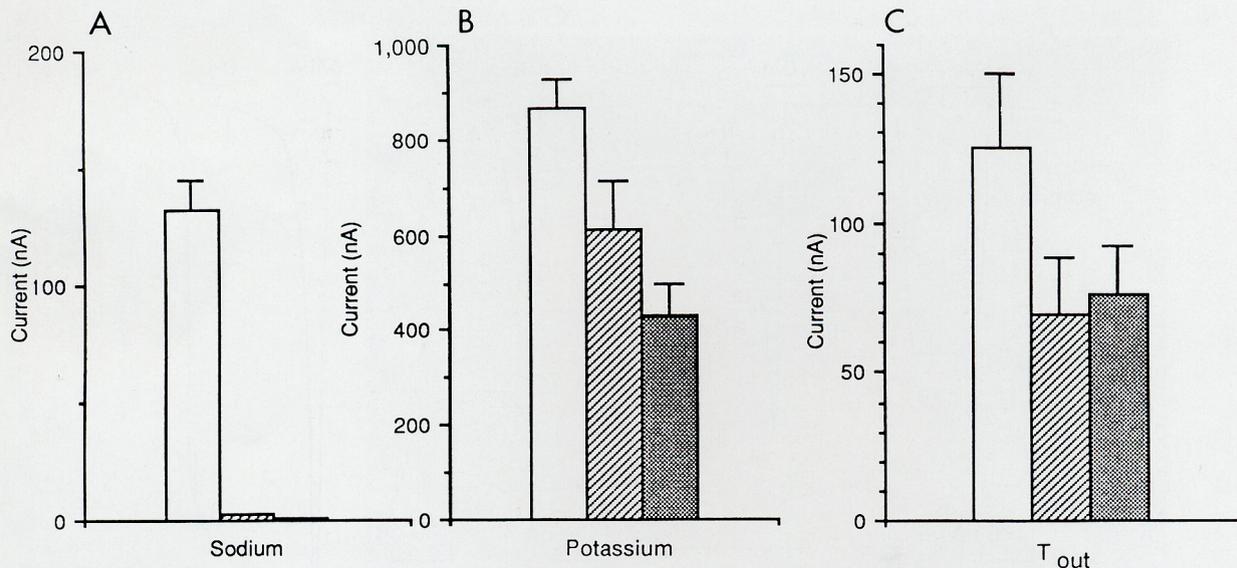


Fig. 1. Voltage-activated sodium (A,B) and potassium (C,D) currents recorded from oocytes injected with c.o.l. mRNA which were untreated (control), or treated with external and intracellular tunicamycin. In this, and Fig. 3, downward deflexions correspond to inward membrane currents. A,B: records at a fast sweep speed, showing the inward sodium current elicited by depolarization from -100 to -10 mV. Each frame shows two superimposed sweeps, elicited by the same depolarizing pulse before and after perfusion with TTX (300 nM). In the control oocyte, the upper trace was recorded after adding TTX, and the difference between the traces gives the magnitude of the TTX-sensitive sodium current (peak = 83 nA in this instance). The two traces superimposed in the tunicamycin treated oocyte, indicating that there was no detectable sodium current. C,D: outward potassium current recorded at a slower sweep speed. Each frame shows currents elicited by depolarization from -100 mV to potentials of (from top to bottom) +40, 0 and -30 mV.

chick



rat

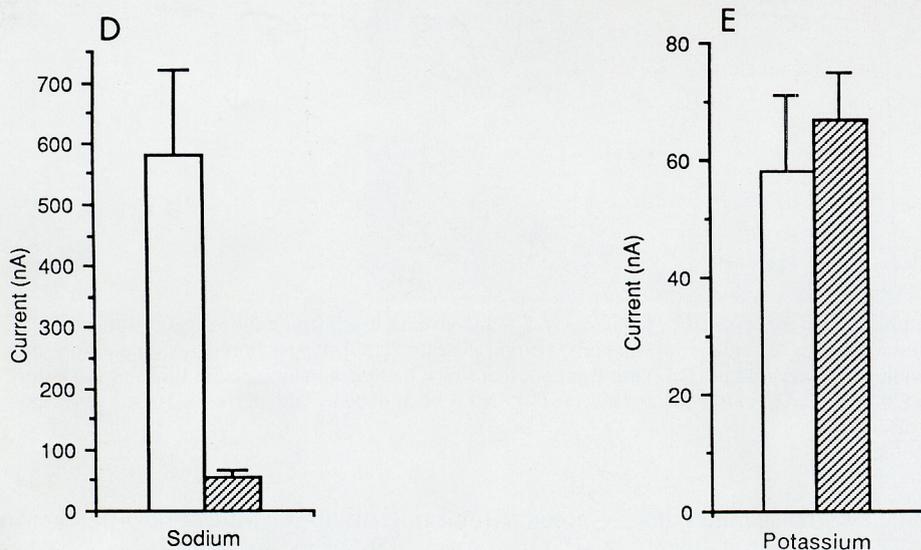


Fig. 2. Mean sizes of voltage-activated currents in control and tunicamycin treated oocytes, which were injected with c.o.l. mRNA (A-C) or rat mRNA (D,E). Sizes of the currents were measured as described in the text and the legend to Fig. 1 and bars indicate 1 S.E.M. Sodium currents were measured at a potential of -10 mV, and potassium currents at potentials of $+40$ mV (B) or -30 mV (D). In each frame (A-C), the left-hand bar gives measurements from control (non tunicamycin-treated) oocytes, the middle bar oocytes treated with external tunicamycin, and the right-hand bar oocytes treated with external and intracellular tunicamycin. Eight control oocytes were examined, and 7 each with external and external plus intracellular tunicamycin. In D and E, the left-hand bars give measurements from control oocytes, while the right-hand bar represents data from the oocytes treated with external tunicamycin. Three control and 4 test oocytes were examined.

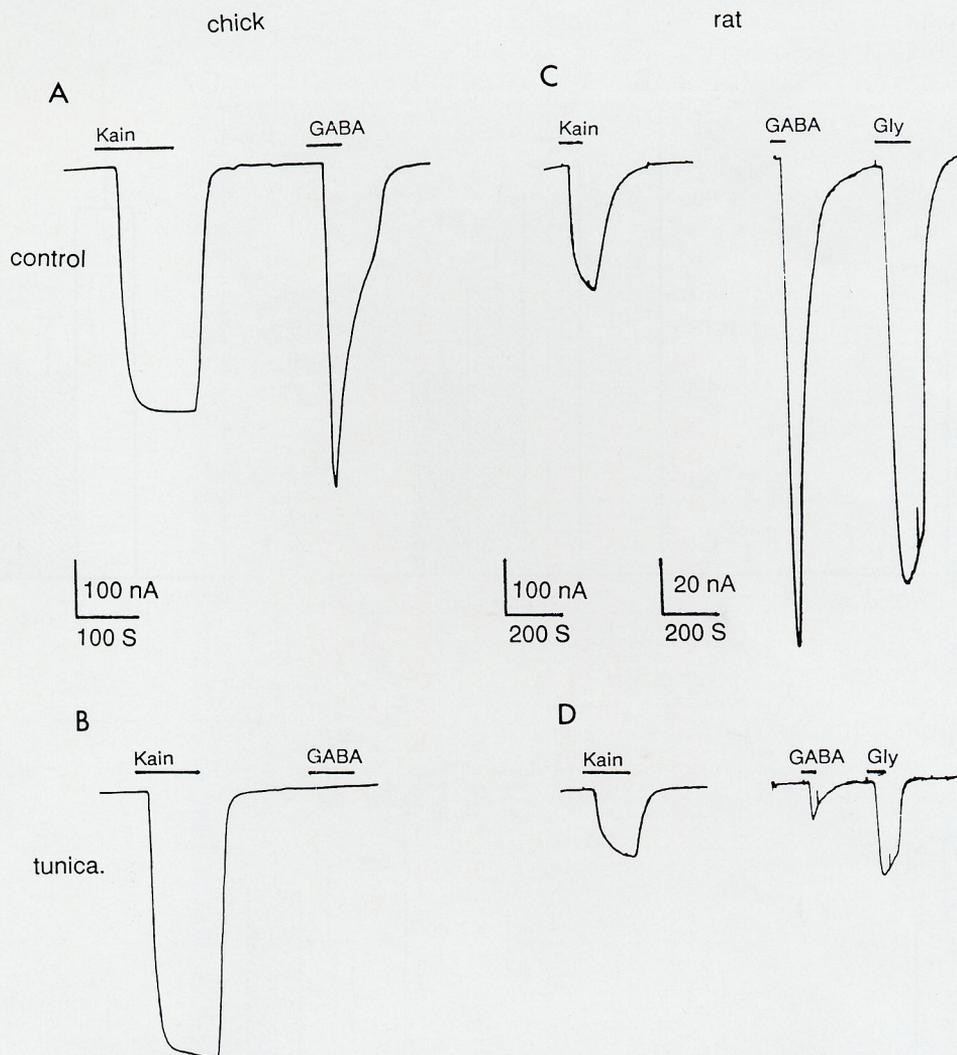


Fig. 3. Effect of tunicamycin on membrane currents evoked by kainate, GABA and glycine. Each frame shows traces obtained from separate oocytes, which were injected with mRNA from c.o.l. (A,B) or rat cerebral cortex (C,D). Upper frames (A,C) are from control oocytes without tunicamycin, while the lower frames (B,D) are from oocytes which had been maintained in tunicamycin before and after mRNA injection. Kainate (10^{-4} M), GABA (10^{-3} M) and glycine (10^{-3} M) were applied by bath perfusion for the times indicated by the bars. Clamp potential was -60 mV.

duces the formation of additional calcium channels in the membrane^{18,27}.

Measurements of the size of the induced sodium current were made by depolarizing the membrane from -100 to -10 mV, with the oocyte bathed first in normal Ringer, and then after adding TTX (300 nM) to the solution. In control (non-tunicamycin treated) oocytes injected with c.o.l. mRNA the traces obtained before and after TTX were different (Fig. 1A), because of the blocking of an inward sodium current. However, injected oocytes that had been ex-

posed to tunicamycin showed little or no difference in the traces after adding TTX (Fig. 1B), indicating that tunicamycin had practically abolished the expression of functional voltage-gated sodium channels.

The mean sizes of the TTX-sensitive sodium current in c.o.l. mRNA injected oocytes from one donor are shown in Fig. 2A, for different treatments with tunicamycin. Oocytes which had been exposed to tunicamycin ($2 \mu\text{g/ml}$) in the incubation solution before and after injection of mRNA gave a mean current only 3% of that in control (non-tunicamycin treated)

oocytes, and the reduction was even greater in those oocytes which had been additionally injected with tunicamycin.

In contrast to the near abolition of the sodium current, the voltage-activated potassium current was less affected by tunicamycin (Fig. 1C,D). The mean size of the potassium current, measured at +40 mV, was reduced by 30% in oocytes treated with external tunicamycin, and by 50% in those with external plus intracellular tunicamycin (Fig. 2B).

The T_{out} current was reduced to about one half of the control value in oocytes treated with tunicamycin (Fig. 2). Interpretation of this result is complicated by the presence of this current in native (i.e. non-mRNA injected) oocytes. However, oocytes from the same donor, which were not injected, or were injected with ineffective mRNA preparations, showed a mean T_{out} current of only 22 nA (± 8 nA S.E.M., 5 oocytes), as compared to 125 nA in control c.o.l. mRNA-injected oocytes and about 70 nA in injected oocytes treated with tunicamycin. Thus, the c.o.l. mRNA considerably enhanced the size of the T_{out} current above that in native oocytes, and this enhancement was reduced, but not abolished, by tunicamycin. The size of the T_{out} current appears not to be limited by the number of calcium-activated chloride channels in the membrane, but rather by the number of voltage-activated calcium channels²⁷.

Effect of tunicamycin on drug-activated channels induced by chick optic lobe mRNA

Oocytes injected with c.o.l. mRNA, and clamped at -60 mV, gave smooth inward membrane currents in response to bath application of GABA and kainate^{23,26,35}. Incubation of oocytes in tunicamycin before and after injection of mRNA greatly reduced their sensitivity to GABA, and some oocytes failed to show any response to GABA, even though they gave responses to kainate which were similar in size to those of control oocytes (Fig. 3).

Mean values of kainate- and GABA-activated currents are shown in Fig. 4 for control and tunicamycin-treated oocytes from one donor injected with c.o.l. mRNA. Incubation with external tunicamycin did not significantly reduce the response to kainate, and treatment with external plus intracellular tunicamycin reduced the response by only about 30%. In contrast, the current activated by GABA was re-

duced by 83% by external tunicamycin, and by 93% by external plus intracellular tunicamycin.

Application of glutamate to oocytes injected with c.o.l. mRNA elicits oscillatory and smooth membrane currents (data not shown), similar to the responses in oocytes injected with rat brain mRNA^{10,35}. Oocytes injected with c.o.l. mRNA which had been treated with external tunicamycin still showed both components of the glutamate-activated current; but while the oscillatory components appeared to be reduced the smooth component was not greatly affected.

Effect of tunicamycin on receptors and channels induced by rat brain mRNA

Experiments similar to those described above were done also on oocytes injected with mRNA derived from adult rat brain. Fig. 3C,D illustrate the effect of tunicamycin on drug-activated currents in these oocytes, and summaries of the data are presented in Figs. 2 and 4. The results with rat brain mRNA-injected oocytes were similar to those obtained with oocytes injected with c.o.l. mRNA (Figs. 2-4). Namely, the voltage-activated sodium current and the GABA-activated current were greatly reduced by tunicamycin, while the potassium current was almost unaffected and the kainate-activated current was reduced by about one third. In addition to these currents, the oocytes injected with rat brain mRNA also showed responses to glycine and serotonin. Glycine activates a smooth chloride current¹¹, while serotonin activates an oscillatory chloride current^{6,12,35}. Treatment with external tunicamycin reduced the response to glycine by about 80% (Figs. 3 and 4). The initial peak of the response to serotonin also appeared to be greatly reduced in the tunicamycin treated oocytes, but the subsequent oscillatory current was less affected (data not shown).

Time dependence of messenger expression and action of tunicamycin

Oocytes from a different donor, injected with c.o.l. mRNA, were incubated with or without tunicamycin for up to 15 days, beginning one day before injection. The oocytes were examined electrophysiologically 3-5 days and 11-15 days after injection. Mean values of kainate- and GABA-activated currents are summarized in Table I for control and tuni-

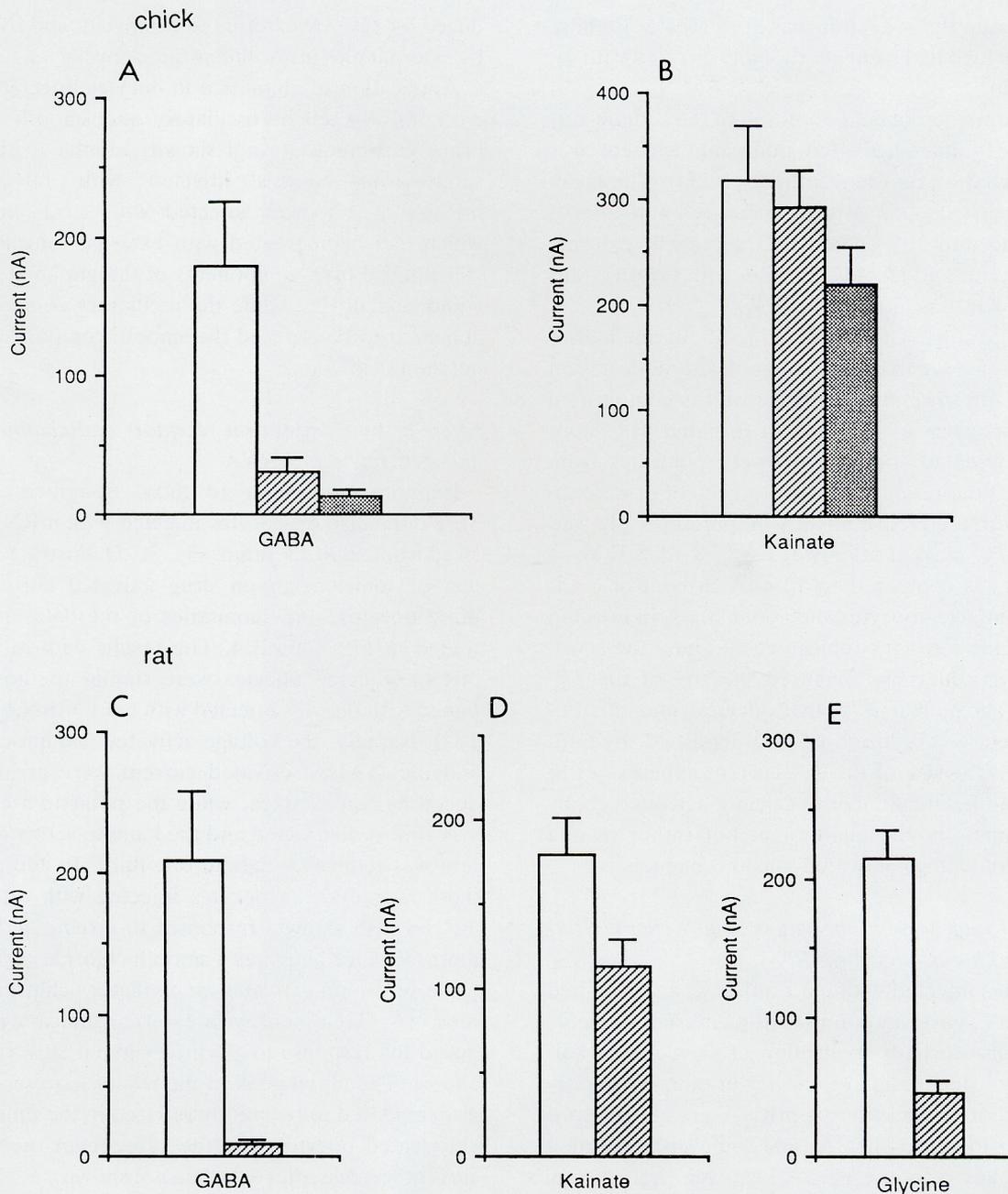


Fig. 4. Effect of tunicamycin on the mean sizes of drug-activated membrane currents in oocytes injected with c.o.l. mRNA (A,B) or rat mRNA (C-E). Responses were measured from records similar to Fig. 3, at a clamp potential of -60 mV, and at concentrations of 10^{-3} M GABA, 10^{-4} M kainate and 10^{-3} M glycine. Data are plotted in the same way as in Fig. 2.

camycin treated oocytes. In this particular batch of oocytes the responses to GABA were significantly smaller (50% or less) in non-tunicamycin treated oocytes after 11–15 days than after 3–5 days, while the kainate-activated current increased about 3-fold over

this time. This suggests that the different receptor proteins and/or the corresponding mRNAs have different stabilities in the oocyte.

Comparison of tunicamycin treated and non-treated oocytes at different times indicated that tuni-

TABLE I

Time-dependent effect of tunicamycin on GABA- and kainate-activated currents induced in oocytes injected with c.o.l. mRNA

Data are given as means \pm S.E.M.

	Current (nA)	
	3–5 Days after injection	11–15 Days after injection
GABA		
Control	634 \pm 136 (n = 8)	192 \pm 112 (n = 4)
Tunicamycin	117 \pm 37 (n = 7)	16 \pm 13 (n = 4)
Kainate		
Control	284 \pm 58 (n = 8)	853 \pm 165 (n = 4)
Tunicamycin	172 \pm 46 (n = 7)	810 \pm 138 (n = 4)

camycin reduced the kainate response by about one third when examined 3–5 days after injection of mRNA, but that there was little difference between treated and non-treated oocytes after 11–15 days. In contrast, the GABA-activated current was strongly reduced by tunicamycin at both 3–5 and 11–15 days (by 82% and 92% respectively).

Recovery after washing tunicamycin

Two oocytes were exposed to external tunicamycin before, and for 5 days after, injection of c.o.l. mRNA, and were then washed in normal Barths solution for 2 days before recording. These oocytes showed a partial recovery of the voltage-activated sodium current, with a mean value of 25 nA, as compared to 4 nA for oocytes exposed to external tunicamycin up to the time of recording. The GABA-activated current did not significantly recover (39 nA as compared to 31 nA). Four other oocytes, which were washed for 2–4 days after treatment with external plus intracellular tunicamycin, showed no appreciable recovery of the sodium or GABA currents.

DISCUSSION

The results demonstrate that the size of different voltage- and drug-operated membrane currents, elicitable in *Xenopus* oocytes following injection of brain mRNAs, is inhibited to different extents by tunica-

mycin. For instance, GABA-activated currents and the voltage-activated Na⁺ current were practically abolished, while the voltage-activated K⁺ and Ca²⁺ currents, and the kainate-activated currents were reduced slightly or not at all. The appearance of sensitivity to glycine was also reduced, but to a lesser extent than that to GABA. The inhibitory effect of tunicamycin on these responses could occur in various ways. For example, there might be a decrease in the number of functional receptors and channels expressed in the oocyte membrane, the non-glycosylated receptors and channels might be degraded more rapidly or, alternatively, the receptor/channel characteristics may be altered so as to give a smaller overall current with receptor numbers remaining unchanged. However, as we have found that non-*N*-glycosylated nicotinic AChRs were accumulated in the intracellular compartments, and were not inserted efficiently in the surface membranes of tunicamycin treated oocytes (unpublished data), we believe that the number of receptors or channels present in the oocyte surface membrane may be reduced by tunicamycin treatment.

It already appears clear that tunicamycin does not block translation of mRNA in the oocyte^{15,19}, since the incorporation of [³⁵S]methionine into newly synthesised proteins was unchanged, and because tunicamycin did not abolish the expression of all receptors and channels. In agreement with this, tunicamycin does not appreciably reduce the translation of mRNAs coding for AChR and Na⁺ channels in cultured cells^{20,32}. Furthermore, in separate experiments we found that the same batch of tunicamycin blocked *N*-glycosylation of the nicotinic AChR in oocytes (K. Sumikawa and R. Mileti, submitted for publication). Thus, it appears that the effects of tunicamycin observed here are specifically due to a blocking of *N*-glycosylation. Carbohydrate moieties might be important in determining the correct conformation of certain receptor and channel molecules, a factor which may be critical in further post-translational modification, intracellular stability, subunit assembly, intracellular transport, membrane insertion and normal function. In this connection it is interesting to note that tunicamycin has been reported either to block the assembly of AChR subunits²⁰ or to increase the rate of degradation of the AChR molecules³¹ in cultured muscle cells. It was also reported

that *N*-glycosylation appears to be required for the subunit assembly and membrane insertion of the Na⁺ channels in cultured brain cells³². However, the requirement of *N*-glycosylation for subunit assembly is not general, since tunicamycin has little effect on the expression of Na⁺-K⁺ ATPase in sensory neurons, even though this consists of two subunits³⁸.

Interpretation of the different degrees to which tunicamycin inhibited the expression of drug- and voltage-operated responses is at present difficult. In experiments similar to those described here, using mRNA derived from *Torpedo* electric organ, tunicamycin reduced the appearance of the functional AChR by about 70–95% (K. Sumikawa and R. Mile-di, unpublished results). However, when a single potential *N*-glycosylation site in the *Torpedo* α -subunit was eliminated by site directed mutagenesis, the expression of functional AChR in oocytes was completely abolished²⁸. Thus, the remaining ACh sensitivity in the tunicamycin treated oocytes may have been due to incomplete blockage of *N*-glycosylation. Similarly, the small remaining sensitivity to GABA and glycine, and the Na⁺ current, in the tunicamycin treated oocytes, may have been due to *N*-glycosylated or partially *N*-glycosylated molecules still expressed in the presence of tunicamycin. Alternatively, non-*N*-glycosylated molecules might be degraded rapidly and the steady-state levels of these molecules in the membrane might be low.

The functional expression of some receptors and channels in the oocytes was not greatly affected by tunicamycin. For example, the kainate-activated current was reduced by at most about 40% 3–5 days after injection and by 11–15 days after injection tunicamycin treated oocytes gave almost identical responses to untreated oocytes. There are several explanations for this result, including the possibility that carbohydrate moieties are not required for the functional expression of the kainate receptor, but the non-*N*-glycosylated receptors are inserted in the oocyte's surface membrane at a slower rate than the *N*-glycosylated receptors. It is interesting to note that the kainate receptor purified from frog brains consists of a single subunit with a molecular weight of 48,000, while glycine and GABA receptors are composed of more than one type of subunit^{13,30,34}.

Finally it should be noted that the effects of tunicamycin on the receptors and channels induced by c.o.l. mRNA were similar to those obtained in oocytes injected with rat brain mRNA. This suggests that the corresponding receptors and channels in the chick optic lobe and rat brain have similar structural features.

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