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Tris Buffer Attenuates Acetylcholine Responses in *Aplysia* Neurons

Abstract. *The commonly used buffering agent tris(hydroxymethyl)methylamine (tris) antagonizes the action of iontophoretically applied acetylcholine on neurons of* Aplysia californica. *Concentrations of 5 to 10 millimolar tris markedly reduced both excitatory and inhibitory responses.*

The buffering agent tris(hydroxymethyl)methylamine (tris) is widely used in biological solutions. In studying synaptic systems in *Aplysia*, we have found that tris at concentrations ranging from 5 to 10 m*M* is a potent inhibitor of both excitatory and inhibitory responses to iontophoretically applied acetylcholine.

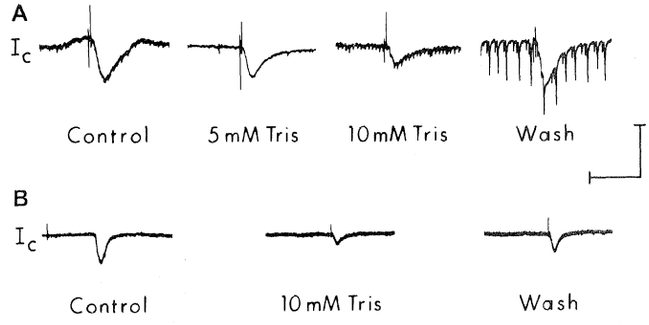
Experiments were performed on neurons found in the pleural and abdominal ganglia of *Aplysia californica*. Ganglia were dissected and pinned, according to standard procedures (*1*), in a bath containing Instant Ocean at *p*H 7.9. Cells were impaled with a single microelectrode for voltage clamping in a manner previously reported (*2*). A second electrode was placed near the cell to iontophoretically apply acetylcholine or dopamine to the neuron under study. To ensure that perfusion or drug action did not alter the position of the iontophoretic electrode relative to the cell, we made frequent visual observations and carefully measured the time to peak of the response. The tris used in these experiments was obtained from Sigma Chemical Company and from Fisher Scientific Co. Several different lots were used.

The data from the first experiment, shown in Fig. 1A, reveal the effect of tris on an excitatory response to iontophoresed acetylcholine. Cell R_{15} was voltage-clamped to −60 mv. Pulses of acetylcholine were applied as the cell was bathed in Instant Ocean at *p*H 7.9. Excitatory current responses are shown. The replacement of the control solution with Instant Ocean containing 5 m*M* tris at *p*H 7.9 attenuated the response. At 10 m*M* tris further reduced the response, and washing for 30 minutes allowed recovery of the current to control levels.

Figure 1B shows data from a similar experiment on an inhibitory response. In this case one of the medial cells from the left pleural ganglion was voltage-clamped to −70 mv (below the reversal potential for the inhibitory postsynaptic response in this cell) and a "reversed" inhibitory (inward) current was recorded as acetylcholine was pulsed. This response was strongly attenuated by 10 m*M* tris, but washing for 15 minutes produced a partial recovery. For both excitatory and inhibitory responses, the control response amplitude could be restored by increasing the strength of the acetylcholine pulse. Hence, the blocking action of tris on acetylcholine responses could easily have been missed in previous studies if the control levels were established in the presence of tris.

We considered the possibility that contamination of the tris was responsible for these effects. However, the experimental results shown above were repeatedly obtained with tris from different lots and different sources. Moreover, these results have been independently confirmed by Parmentier and Zbicz (*3*). Using the land snail *Helix aspersa*, they also observed that 10 m*M* tris produced marked attenuation of an excitatory response to iontophoresed acetylcholine.

The time required for tris to act varied somewhat from experiment to experiment. Measurable effects were often seen after 1 minute, but on some occasions they took up to 10 minutes. The abdominal ganglion neurons are invaginated by glial processes and are encased in a connective tissue sheath (*4*), both of which may reduce the availability of charged molecules to the neuronal surface. Since the pK_a of tris is 8.3 (K_a is the association constant), solutions near *p*H 8.3 contain both charged and uncharged forms of tris. Solutions with a higher *p*H contain more of the uncharged form, which might facilitate penetration.



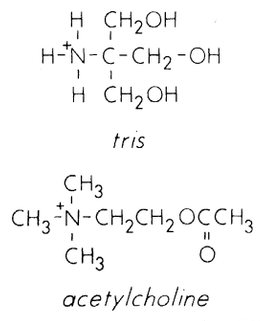


Fig. 1 (left). Voltage clamp data showing currents following iontophoresis of acetylcholine. Inward (depolarizing) currents are downward deflections. (A) Current underlying acetylcholine excitation of abdominal ganglion cell R_{15} clamped to −60 mv. Attenuated responses are obtained with 5 and 10 m*M* tris. Washing for 30 minutes restores the control response and elicits increased spontaneous synaptic activity. (B) Current underlying acetylcholine-induced inhibition of a neuron in the left pleural ganglion. This neuron was clamped to − 70 mv (below the reversal potential for the inhibitory response in this cell) and thus inward, rather than outward, currents are seen. Tris at 10 m*M* strongly attenuates the response, and washing for 15 minutes partially reverses the effect. Calibration: 15 seconds and 15 na in (A) and 36 seconds and 6 na in (B). Fig. 2 (right). Chemical structures of tris and acetylcholine. Tris is shown in its charged form.

We tested the effect of tris solutions at *p*H values ranging from 7.0 to 9.0. In all experiments tris was increasingly effective as the *p*H was increased.

The results of these experiments suggest that tris is more effective in the uncharged form, but they do not indicate whether it is working at an intracellular or an extracellular site, or, in fact, whether the tris is charged or uncharged at that site.

Tris could be entering the cell and interfering with the processes that occur after transmitter-receptor interaction. On the other hand, the chemical similarity of tris to acetylcholine (see Fig. 2) suggests that tris could be an antagonist of acetylcholine binding to its receptor.

Tris may be a specific antagonist of cholinergic responses. We have performed preliminary experiments with iontophoresed dopamine which show that excitatory responses are minimally altered, if at all, by 10 m*M* tris. In the case of 5-hydroxytryptamine (5-HT), Gerschenfeld and Paupardin-Tritsch (*5*) used tris to substitute for sodium in a study of 5-HT-mediated hyperpolarizations and did not report any difficulty. In contrast, Blankenship *et al.* (*6*) used tris (and occasionally choline chloride and sucrose) as a substitute for sodium in the study of cholinergic excitation in *Aplysia*. Only in a few experiments were they able to demonstrate the expected shift in reversal potential. In the remainder, the response was nearly abolished and estimates of reversal potential could not be made. It is not clear from (*6*) which sodium substitute was used in the various experiments. The abolition of the response may have been caused by direct action of tris on the acetylcholine-mediated excitation.

The results reported here and the confirming results of Parmentier and Zbicz (*3*) were obtained with molluscan systems; further studies should be carried out with cholinergic responses in other systems. Since a number of laboratories have utilized tris-buffered solutions in both biochemical (*7, 8*) and physiological (*9*) studies of cholinergic systems, specific experiments to explore the effect of tris on acetylcholine binding and the associated conductance changes would be most helpful. Until the full extent of the synaptic actions of tris is known, it should be used only after proper control experiments have been performed.

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Design of Specific Inhibitors of Angiotensin-Converting Enzyme: New Class of Orally Active Antihypertensive Agents

Abstract. *A hypothetical model of the active site of angiotensin-converting enzyme, based on known chemical and kinetic properties of the enzyme, has enabled us to design a new class of potent and specific inhibitors. These compounds, carboxyalkanoyl and mercaptoalkanoyl derivatives of proline, inhibit the contractile response of guinea pig ileal strip to angiotensin I and augment its response to bradykinin. When administered orally to rats, these agents inhibit the pressor effect of angiotensin I, augment the vasodepressor effect of bradykinin, and lower blood pressure in a model of renovascular hypertension.*

The nonapeptide SQ 20,881, <Glu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (*1*), has been shown to be a specific and potent inhibitor of angiotensin-converting enzyme in vitro (*2*) and in vivo (*3*). Preliminary clinical studies (*4*) have demonstrated the great potential of SQ 20,881 as a novel antihypertensive drug, limited only by its lack of oral activity. This therapeutic potential led us to test other structural types in our search for new, specific, and orally active inhibitors.

Specific inhibitors of angiotensin-converting enzyme would be expected to show the profile of activities exemplified by SQ 20,881 (Table 1) with respect to inhibition of the isolated enzyme (*5*), inhibition of the contractile response of smooth muscle to angiotensin I (*2*), augmentation of the contractile response to bradykinin (*2, 6*), and no effect on responses to other agonists. Only a small fraction of the nonpeptidic compounds that we have tested were potent inhibitors of angiotensin-converting enzyme, and very few of these showed the desired specificity. However, accumulated knowledge of the chemical and enzymatic properties of angiotensin-converting enzyme suggested the possibility of a new approach, the rational design of specific inhibitors of angiotensin-converting enzyme.

Previous studies with substrates and inhibitors of angiotensin-converting enzyme (*7*) suggested that this peptidase was a carboxypeptidase similar to pancreatic carboxypeptidase A, even though it releases dipeptides rather than single amino acids from the carboxylic acid end

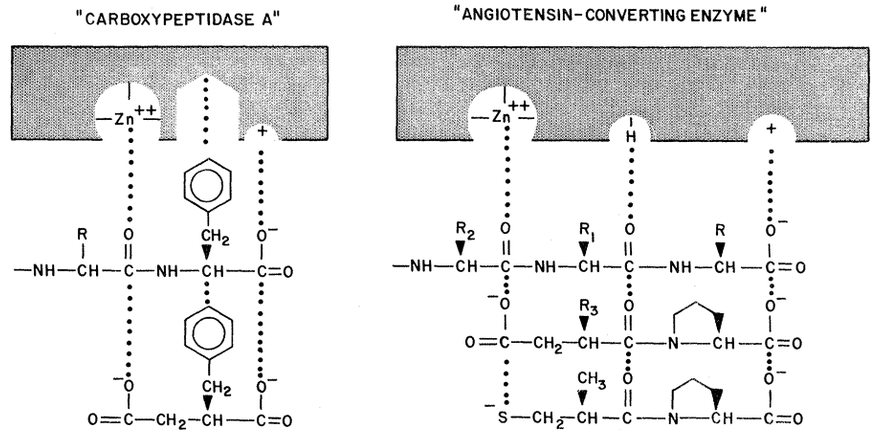

Fig. 1. Schematic representation of the binding of substrates and inhibitors at the active site of pancreatic carboxypeptidase A, and at the hypothetical active site of angiotensin-converting enzyme.