the parapodia. The abdominal ganglion, which contains the siphon sensory and motor neurons, was partially desheathed, and the preparation was thoroughly washed with normal artificial seawater (Instant Ocean) before the experiment was begun.

19. Standard electrophysiological techniques were used (*7–9*). Sensory neurons and motor neurons were impaled with single-barreled glass microelectrodes filled with 2.5*M* potassium chloride and beveled to a resistance of 10 to 20 megohms. A Wheatstone bridge circuit was used for recording while passing current either to depolarize neurons (the sensory cells) or to hyperpolarize them (the motor neuron). In experiments in which the motor neuron was hyperpolarized to prevent it from firing, it was held at the same level while testing the PSP's from the two sensory neurons before and after training.
20. W. N. Frost, V. F. Castellucci, E. R. Kandel, in preparation.
21. The siphon sensory neurons are a cluster of about 24 cells that have similar properties and synaptic connections (*7*). These cells do not receive conventional excitatory synaptic input from each other or from the US (tail shock). Two neurons were picked arbitrarily and assigned to be paired or unpaired in such a way as to balance the average amplitudes of the paired and unpaired EPSP's before training (4.2 ± 0.7 mV for the paired neurons and 4.9 ± 0.8 mV for the unpaired neurons). Typically each sensory neuron was made to fire a train of five action potentials by intracellular injection of 40- to 50-msec depolarizing current pulses at 10 Hz. Current intensity was adjusted so that each current pulse produced one action potential. Two types of US were used: either 1.5 seconds of 50 mA, 60-Hz, a-c shock delivered to the tail through bipolar capillary electrodes, or a 1.5-second, 10-Hz train of 3-msec pulses delivered to the posterior pedal nerves through bipolar Ag-AgCl electrodes. The intensity of the tail or posterior pedal nerve stimulation was sufficient to produce brisk firing in the postsynaptic neuron when it was not hyperpolarized. Onset of the US coincided with offset of intracellular stimulation of the paired neuron and followed stimulation of the unpaired neuron by 2.5 minutes.
22. The summary statistics given are means and standard errors of the means. In all cases statistical comparisons are *t*-tests for correlated means. Thus each experimental score was compared to a control value in the same experiment. For within-cell comparisons, the control value was the pretest score, while for between-cell comparisons, the control value was the score for the other cell in the experiment. The EPSP's from the paired and unpaired neurons were tested 2.5 minutes apart, either 5 or 15 minutes after the last US. The order in which the two PSP's were tested was counterbalanced. Only the monosynaptic component of the EPSP (as judged by short and constant latency and smooth rise) was measured, although additional changes were sometimes observed in the polysynaptic response.
23. The degree of correlation between the cellular data and the behavioral data is better than might be expected, since in one case we measured EPSP amplitude and in the other case we measured duration of siphon withdrawal. However, a good correlation between these two measures has also been observed in previous experiments [for example, T. J. Carew and E. R. Kandel, *Science* **182**, 1158 (1973)] and could be explained by several possible mechanisms: (i) Frost *et al.* (*20*) identified interneurons in the siphon withdrawal circuit that fire an extended train of spikes in response to brief excitatory synaptic input from the sensory neurons. An increase in the amplitude of synaptic potentials from the sensory neurons could produce an increase in the duration of the resulting train of spikes in some of these interneurons, thus producing an increase in the duration of siphon withdrawal. (ii) The same cellular change that underlies increased transmitter release from the sensory neurons (for example, a decrease in K^+ conductance) could also lead to repetitive firing of the sensory neurons in response to a brief tactile stimulus. (iii) The activity-dependent change that occurs in the sensory neurons could occur in some interneurons as well, and might prolong interneuronal firing in a similar fashion. These possibilities are neither mutually exclusive nor exhaustive.
24. The abdominal ganglion was placed in a small well and perfused with TEA solution while the rest of the preparation was perfused with normal seawater. The pleural-abdominal connectives were led through a silicon grease seal under the walls of the well. Sensory neurons were assigned to be paired or unpaired in such a way as to balance the durations of their action potentials before training (71 ± 9 msec for the paired neurons and 77 ± 10 msec for the unpaired neurons). Intracellular stimulation consisted of a single 5-msec depolarizing pulse. This typically produced a burst of several spikes in the sensory neuron, of which only the first was measured. The value of the spike duration for the pretest and each of the posttests was the mean of three measurements taken at 5-minute intervals. The US was 1.5 seconds of 50 mA, 60-Hz a-c shock to the tail. Onset of the US followed intracellular stimulation of the paired neuron by 500 msec and followed stimulation of the unpaired neuron by 2.5 minutes
25. Continued broadening of the action potentials may have a number of possible explanations including (i) the onset of a process of consolidation of the memory into a long-term form, (ii) recovery from spike narrowing caused by repeated stimulation of the sensory neurons during training, (iii) prolonged exposure of the neurons to TEA, or (iv) progressive deterioration of the preparation. Preliminary experiments indicate that US-alone training does not produce continued broadening, which suggests that it may be due to recovery from spike narrowing.
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32. A synaptically induced long-lasting decrease in K^+ conductance in an interneuron or motor neuron would lead to (i) a long-lasting increase in the ease with which the neuron could be excited by synaptic input [T. J. Carew and E. R. Kandel, *J. Neurophysiol.* **40**, 721 (1977)], and (ii) a long-lasting increase in the firing rate or bursting frequency of a spontaneously active neuron. A consequence of the mechanism we are proposing is that these increases would be amplified if the neuron happened to be firing action potentials at the time of the modulatory synaptic input. This result would correspond formally to (and could underlie) an increase in the probability, rate, or frequency of a behavior by contingent reinforcement of that behavior.
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7 October 1982

Associative Conditioning of Single Sensory Neurons Suggests a Cellular Mechanism for Learning

Abstract. *A cellular analog of associative learning has been demonstrated in individual sensory neurons of the tail withdrawal reflex of* Aplysia. *Sensory cells activated by intracellular current injection shortly before a sensitizing shock to the animal's tail display significantly more facilitation of their monosynaptic connections to a tail motor neuron than cells trained either with intracellular stimulation unpaired to tail shock or with tail shock alone. This associative effect is acquired rapidly and is expressed as a temporally specific amplification of heterosynaptic facilitation. The results suggest that activity-dependent neuromodulation may be a mechanism underlying associative information storage and point to aspects of subcellular processes that might be involved in the formation of neural associations.*

The use of intracellular techniques to investigate neuronal changes produced by classical and operant conditioning paradigms (*1*) encourages the belief that mechanisms of associative information storage can be analyzed on the cellular level. Because associative learning is usually quite sensitive to motivational and attentional factors, some psychologists have assumed that the formation of associations depends, in part, upon the contiguous activation of sensory "analyzers" and modulatory "arousal centers" (*2*). We have tested this general idea on the neuronal level in the mollusk *Aplysia californica* by examining the associative interaction of electrophysiological activity in individual sensory neurons with neuromodulatory concomitants of defensive arousal. Our results suggest a cellular mechanism for associative information storage, activity-dependent neuromodulation, that may be of general significance.

We applied a cellular analog of a differential classical conditioning procedure simultaneously to three mechanoafferent neurons—which innervate the tail (*3*)—in the left pleural ganglion of each animal. Because noxious tail stimulation produces defensive arousal and modulates (heterosynaptically facilitates) (*3, 4*) synaptic connections of these sensory neurons, we used tail shock as the reinforcing or unconditioned stimulus (US) (*5*). Training and testing were conducted in a reduced "split-foot" preparation (*6*). Associative and nonassociative effects of training were measured by testing the amplitude of single monosynaptic excit-

atory postsynaptic potentials (EPSP's) evoked in a common postsynaptic tail motor neuron (*3, 7*) by brief intracellular stimulation of each sensory cell. These tests (*8*) were applied at 5-minute intervals before (pretest), during, and after (posttest) training.

After the pretest phase, the sensory neurons were assigned (*9*) to one of three training procedures: paired (CS+; $N = 10$), unpaired (CS−; $N = 10$), or sensitization (Sens; $N = 9$) (Fig. 1A). The paired and unpaired conditioned stimuli (CS+ and CS−) consisted of a brief suprathreshold train of nine depolarizing current pulses injected through the intracellular electrode (*10*). This spike train mimicked the response of these sensory neurons to moderate mechanical stimulation of the skin (*3*). The cells receiving Sens training were exposed only to the effects of the US. Each trial began with delivery of the CS+ to the paired sensory neuron (Fig. 1A); 600 msec later, the US was applied to the tail; and after 2 minutes the CS− was applied to the unpaired sensory neuron. After an additional 2 minutes (1 minute before the next trial), all three sensory neurons were tested with single depolarizing pulses (*8*) to elicit EPSP's in the motor neuron. Five training trials were given (with four interpolated tests), followed by at least six additional tests.

Figure 1A illustrates the neural concomitants of each type of training, showing the sensory cell responses and the synaptic responses in the motor neuron to the CS+, CS−, and US. In this animal, CS+ produced more facilitation of the monosynaptic EPSP than did CS− or Sens training (Fig. 1, B and C).

Figure 2A (top) shows the pooled EPSP amplitudes in each group (ten animals and 29 cells) (*11*). A treatment-by-trials analysis of variance on the ten tests given during the training and posttest phases revealed overall significant effects of both type of training and number of trials ($F_{2,26} = 3.77$, $P < .05$; $F_{9,234} = 27.64$, $P < .01$). Subsequent pairwise comparisons at two selected tests were performed with the Newman-Keuls procedure. At test 5 (5 minutes after the last US) the CS+ cells showed significantly more synaptic facilitation than either the CS− or Sens cells ($P < .05$); there was no difference between the CS− and Sens cells. By contrast, at test 10 there were no significant differences among these groups.

The US alone produces nonspecific heterosynaptic facilitation of the sensory neurons (Sens, Fig. 2A, top). It seemed likely that variability in the amount of nonspecific facilitation produced by the US in different animals partially obscured the associative effect specific to the pairing of spike activity with the US. For example, on test 10 the mean EPSP amplitude of Sens cells in different animals ranged from 60 percent to 166 percent of their mean pretest levels, and similar variability was seen in CS+ (89 to 251 percent) and CS− (35 to 272 percent) EPSP's. Nevertheless, nine of ten animals showed greater facilitation (or less depression) in CS+ cells than in CS− cells at test 10. To reduce the effects of variability among different animals in nonspecific facilitation, we used the Sens cell in each animal as an index of the amount of nonspecific facilitation in that animal and estimated the magnitude of the associative effect relative to the nonspecific facilitation on each test. Thus, for each cell we normalized all EPSP's to the mean of the three baseline tests in the pretest phase. In each animal ($N = 9$), we then divided the normalized EPSP amplitudes of the CS+ and CS− cells on each test by the normalized Sens EPSP amplitude on that test to obtain CS+ and CS− facilitation ratios (Fig. 2A, bottom). A facilitation ratio of 1 indicates facilitation equal to that produced by sensitization alone, whereas facilitation ratios greater than 1 indicate synaptic facilitation greater than expected from sensitization alone.

A treatment-by-trials analysis of variance on the facilitation ratios of CS+ and CS− cells in the training and posttest phases revealed overall significant effects of type of training ($F_{1,16} = 10.14$, $P < .01$) but not of number of trials ($F_{9,144} = 1.58$). Subsequent Newman-Keuls tests revealed that CS+ test responses were significantly greater than CS− responses 5 minutes (test 5, $P < .05$) and 30 minutes after the last US (test 10, $P < .01$). In three of the animals, the sensory cells were held for over 75 minutes, and in each case, the CS+ cell showed more facilitation than the CS− cell. Thus the associative effect appears to be long-lasting. In addition, the nearly constant value of the CS+ facilitation ratio throughout the training and posttest phases (Fig. 2A, bottom) suggests that the associative process produces a nearly constant amplification of the nonspecific synaptic facilitation from the US on each trial.

The associative change is not due to a generalized change in the properties of

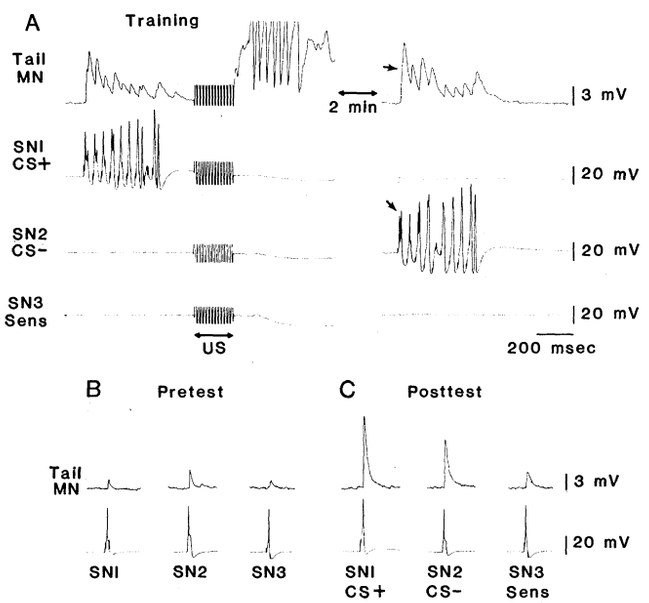

Fig. 1. Associative conditioning of a sensory neuron. Three tail sensory neurons (*SN*) and a tail motor neuron (*MN*) were examined simultaneously. (A) Training procedure (illustrated by trial 3). Each CS consisted of nine intracellular suprathreshold depolarizing pulses. SN1 received the CS+ (which elicited 11 spikes on this trial) 600 msec before the US (tail shock artifacts visible), and SN2 received the CS− (which elicited 12 spikes) 2 minutes later. The US was applied outside of the excitatory receptive fields of the sensory neurons examined. The resulting spikes in the motor neuron are clipped by the pen recorder. SN3 (Sens) received no CS, providing an index of nonspecific heterosynaptic facilitation. The first pulse during the CS− elicited a double spike in SN2 (arrow), causing summation of the resulting EPSP's in the motor neuron (arrow). (B) Monosynaptic test responses during the pretest (test B3). (C) Monosynaptic test responses during posttest (test 6, 10 minutes after training). The test response of SN1 (CS+) showed more facilitation (732 percent of baseline) than did SN2 (CS−, 254 percent) or SN3 (Sens, 214 percent) in this animal.

the postsynaptic cell since, in each animal, a single motor neuron manifested simultaneously the alterations produced by each of the three cellular training protocols. Alternatively, the association may occur within the modulatory system mediating the heterosynaptic facilitation. This would require nonoverlapping modulatory subsystems specific to each sensory neuron, an unlikely arrangement since focal stimulation of points on the tail (activating few tail sensory neurons) causes heterosynaptic facilitation of all the tail sensory neurons (*3*). Another possibility is that the associative locus is the sensory neuron itself. Support for this possibility comes from recent findings of changes in membrane potential and input resistance of the sensory cell soma produced by pairing (*12*).

These results suggest a cellular mechanism for storing associative information, namely, activity-dependent neuromodulation (Fig. 2B). We propose that a motivationally significant US causes the diffuse release (synaptic or humoral, or both) of substances that modulate the functional strength of various neurons. In the tail withdrawal reflex, this neuromodulation is expressed as heterosynaptic facilitation—and apparently an increase in excitability (*3, 12, 13*)—of the sensory neurons. Associative specificity occurs because the degree of neuromodulation depends on the timing of prior electrophysiological activity in the modulated cells, a dependence similar to the permissive effect of spike activity postulated by Kandel and Tauc (*14*). However, rather than acting permissively, spike activity immediately before a neuromodulatory signal from a US appears to cause a powerful amplification of the amplitude and duration of the modulatory effects, an amplification that does not occur if the same change in activity is separated in time from the modulatory signal.

Our proposed associative mechanism differs from many cellular models of associative learning in not being dependent on the concurrent activation of pre- and postsynaptic elements at particular synapses (*15*). One consequence is that this mechanism could be used flexibly for different associative effects in different neuronal systems. For example, in a sensory system, this mechanism could allow an organism to learn to attend selectively to previously insignificant stimuli without necessarily linking these stimuli to particular responses.

A subcellular mechanism for implementing the associative change is suggested by properties of mechanoafferent neurons in *Aplysia*. The modulatory effects of the US have been linked to the activity of adenosine 3′,5′-monophosphate (cyclic AMP) in *Aplysia* siphon sensory neurons (*16*), and evidence now suggests a similar role in the tail sensory neurons (*12, 17*). Indirect evidence suggests an elevation of Ca^{2+} levels in tail sensory cells produced by the CS (*18*). Therefore, a simple, testable hypothesis is that one major intracellular messenger (Ca^{2+}) amplifies the effects of the other (cyclic AMP), perhaps through the activation of an adenylate cyclase by Ca^{2+} (*19*), or through synergistic effects of Ca^{2+}-dependent and cyclic AMP–dependent kinases (*20*) in the CS+ cell.

Because activity-dependent neuromodulation can selectively associate diffuse modulatory signals with functionally active target cells, this mechanism would seem to be useful for a variety of plastic processes. Its potential generality is further suggested by similar findings obtained independently in siphon sensory neurons of *Aplysia* (*21*) and by results consistent with activity-dependent and Ca^{2+}-dependent neuromodulation in mammalian cortical and hippocampal cells (*22*).

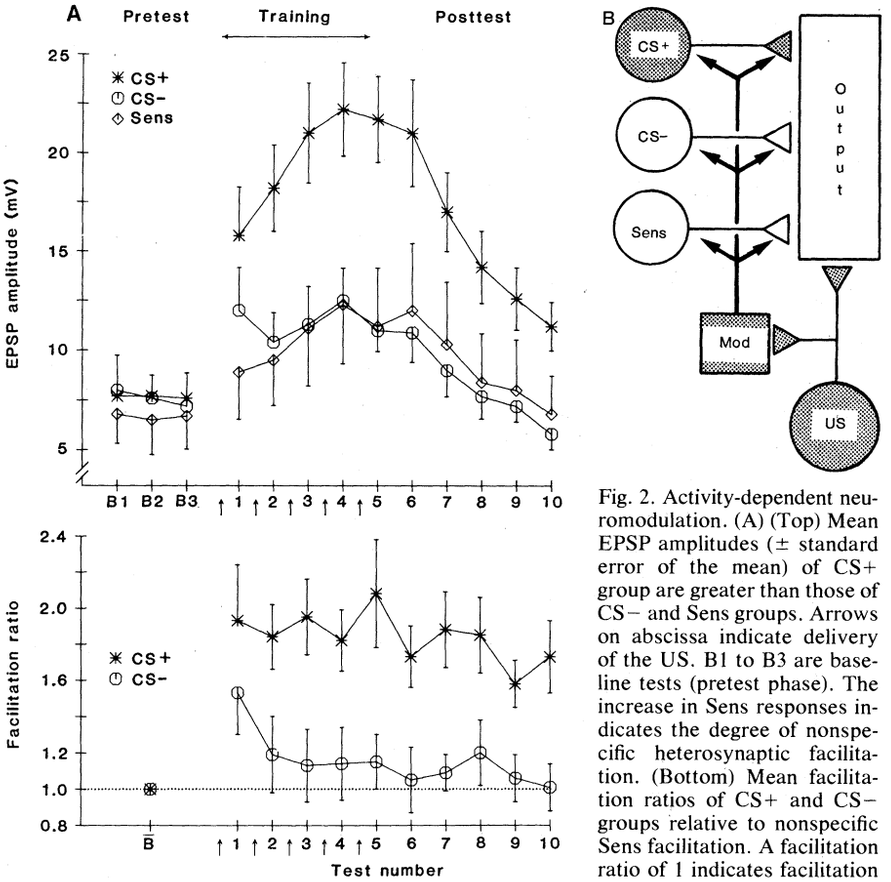

Fig. 2. Activity-dependent neuromodulation. (A) (Top) Mean EPSP amplitudes (± standard error of the mean) of CS+ group are greater than those of CS− and Sens groups. Arrows on abscissa indicate delivery of the US. B1 to B3 are baseline tests (pretest phase). The increase in Sens responses indicates the degree of nonspecific heterosynaptic facilitation. (Bottom) Mean facilitation ratios of CS+ and CS− groups relative to nonspecific Sens facilitation. A facilitation ratio of 1 indicates facilitation equal to that expected from sensitization alone. The CS+ cells show temporally specific, activity-dependent amplification of US-evoked facilitation. (B) General model of activity-dependent neuromodulation. Stippling indicates temporally contiguous activity. A motivationally potent US both activates a neural output system and diffusely modulates (*Mod*) afferents to the output system. Increased spike activity in the CS+ afferent immediately before the modulatory signal amplifies the degree and duration of the modulatory effects, perhaps through the Ca^{2+} sensitivity of a US-evoked second messenger. In turn, by increasing transmitter release or excitability of the CS+ cell; or both (*13*), the amplified modulatory effects strengthen the functional connection between the CS+ cell and the output system.

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7. Three presumptive motor neurons were used: LP5, LP6, and LP7. These cells were identified by their motor effects on the tail, and their size, location, and electrophysiological characteristics (*3*). During each test the motor neuron was hyperpolarized to the same membrane potential (about 25 mV from the initial pretest level) with a second intracellular electrode to prevent spike initiation.
8. EPSP's elicited by single action potentials at regular 5-minute intervals (except in trial 5 where the interval was 6 minutes) were used to assay associative and nonassociative effects of training rather than using the CS itself. This procedure minimized the effects of testing on the acquisition and retention of associative effects, and allowed the examination of the effects of the US alone (Sens responses) during training.
9. For the first five animals, a coin flip was used to decide which of the two cells with EPSP's closest to each other in amplitude was to be CS+, and which CS−, with the remaining cell used as Sens. For the remaining five animals, CS+ and CS− assignments were made so as to minimize the difference between the CS+ and CS− groups in their mean pretest scores (Figure 2A, top). No obvious differences in the effects of training were observed between the first and second five animals.
10. Constant-current pulses lasting 20 msec were applied in 400-msec trains (20 Hz) through the recording electrode (2 to 6 megohms) (WPI capillaries TW150F). Current intensity (about 4 nA) was adjusted to about twice the spike threshold before the pretest. Since sensitizing stimulation often changes the excitability of the sensory neurons (*3*, *12*), current intensity was sometimes readjusted during training to maintain a relatively constant number of spikes in response to each CS. Differences in CS intensity cannot account for differences between CS+ and CS− effects since in the overall study the average number of spikes evoked by the CS on each trial was the same in each group (9.1 ± 1.9 and 9.1 ± 2.3, respectively, mean ± standard deviation).
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Methyl 4,6-Dichloro-4,6-Dideoxy-α-D-Galactopyranoside: An Inhibitor of Sweet Taste Responses in Gerbils

Abstract. *The sugar methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside (DiCl-gal) is a new type of inhibitor of the gerbil's electrophysiological taste response to sucrose or saccharin. Saturated solutions of this compound alone barely stimulate the gerbil's taste nerve. But, when mixed with sucrose or saccharin, DiCl-gal suppresses the gerbil's taste response to these two sweeteners. In contrast, when mixed with sodium chloride or hydrochloric acid, DiCl-gal does not affect the taste responses to these compounds. However, unlike other inhibitors of sweet taste, the DiCl-gal taste suppression is short-lived and occurs only when the inhibitor is combined with the sweetener.*

My colleagues and I have been investigating the electrophysiological taste responses of gerbils to sugars and artificial sweeteners (*1–3*), seeking inhibitors that would provide some insight into the mechanism of the sweet taste response. We discovered a new type of inhibitor when we were comparing the taste responses to sucrose with those to tetrachloro-*galacto*-sucrose (4,6-dichloro-4,6-dideoxy-α-D-galactopyranosyl 1,6-dichloro-1,6-dideoxy-β-D-fructofuranoside). This chlorine-containing sucrose derivative is about 100 times as effective as sucrose in producing an electrophysiological response (*2*, *4*). Therefore, in an attempt to produce a "super sweetener," we synthesized methyl 4,6-dichloro-4,6-dideoxy-α-D-galactopyranoside (DiCl-gal), a monosaccharide that contains chlorine atoms and also closely resembles the chlorinated sucrose derivative. This sugar barely stimulated the gerbil's taste receptors even at a concentration of 0.1*M*, a saturated solution. In our earlier research, whenever we found

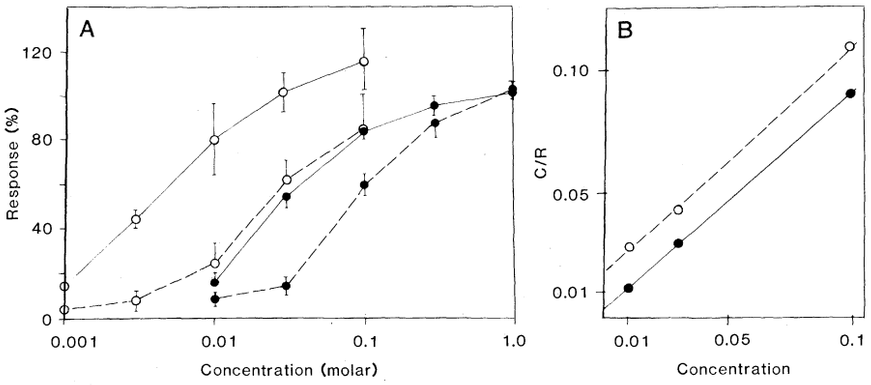

Fig. 1. (A) A comparison of the mean integrated nerve responses to sweetener solutions with the responses to mixtures of the sweetener and DiCl-gal. The sucrose solutions were prepared with deionized water: ●——●, sucrose ($N = 5$); ●– – –●, mixture of sucrose and 0.1*M* DiCl-gal ($N = 5$). The H-saccharin solutions were prepared with 0.1*M* potassium phosphate buffer, *p*H 7 : ○——○, H-saccharin ($N = 4$); ○– – –○, mixture of H-saccharin and 0.1*M* DiCl-gal ($N = 4$). The bars indicate ± 2 standard errors. (B) A Beidler plot of integrated nerve responses to H-saccharin (●) and mixtures of H-saccharin and 0.1*M* DiCl-gal (○); *C* represents concentration and *R* is response.