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18. We thank E. Freier, D. Tukey, and B. Hedlund for electrophoretic analyses, M. Leida, J. Carlsson, and J. Haldane for helpful discussions, and D. Konzen for help with manuscript preparation. J.W.E. is the recipient of an NIH research career development award. This research was conceived, initiated, and fully supported by Evreka, Inc., Flanders, N.J., and is the subject of a pending application for U.S. Letters Patent by the inventor and assigned to Evreka, Inc. This report was presented in abstract form in *Blood* **58** (Suppl. 1), 26 (1981).

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Primary Changes of Membrane Currents During Retention of Associative Learning

Abstract. *A single identified neuron was repeatedly isolated by axotomy from the central nervous system of the nudibranch mollusk* Hermissenda crassicornis. *An early voltage-dependent outward K^+ current of this neuron was reduced and more rapidly inactivated for animals previously trained with paired but not randomized light and rotation. Since this current change can affect interneuron and motorneuron output via known synaptic pathways, it helps explain a long-lasting behavioral change that shows the defining features of vertebrate associative learning.*

During the daylight period of its light-dark cycle the nudibranch mollusk, *Hermissenda crassicornis* moves toward a light source, that is, up a light-intensity gradient (*1*). After 1-hour training sessions with paired light and rotation on three successive days, this positive phototaxis is markedly reduced (*1, 2*). This behavioral change increases as a function of practice, persists for several days, and is pairing-specific (that is, does not follow randomly associated light and rotation) and is stimulus-specific (*2, 3*). Changes of individual neurons were found to be closely correlated with the behavioral change (*3–5*), which shares defining features of associative learning as demonstrated for vertebrates. The data available indicated that primary (*6, 7*) neuronal changes occurred within the type B photoreceptors (of which there are three in each eye). After axotomy, which eliminated all impulse activity and synaptic interactions, type B cells from animals trained with paired light and rotation (but not control procedures) showed an increased input resistance and an enhanced depolarizing response after light steps (*3–5*).

We hypothesized that these changes of type B properties, observed 1 and 2 days after the last days of associative training, could be due to long-term reduction of voltage-dependent K^+ currents across the type B photoreceptor membrane (*4, 5, 8*). Two such currents were determined (*9*) for this membrane (in the absence of light): an early, rapidly inactivating voltage-dependent K^+ current (I_A) [compare (*10*)] and a late, slowly inactivating voltage-dependent K^+ current (I_B) [compare (*11*)]. It has been possible to separate these currents pharmacologically: I_A is preferentially blocked by 10 m*M* 4-aminopyridine and I_B by tetraethylammonium ion (*12*). Residual I_{Na} or $I_{Ca^{2+}}$ were not detected (for the dark membrane) after treatments that would eliminate K^+ currents, enhance inward currents, or both.

We have tested the hypothesis of a reduced I_A or I_B during retention of the associatively learned behavior. Using a blind (*13*) experimental procedure, type B neurons axotomized immediately before recording were removed from animals previously exposed to training with paired light and rotation ("paired" group) or with randomly associated light and rotation ("random" group). These neurons were voltage-clamped at −60 mV holding potential with two microelectrodes (*8, 14*) 1 and 2 days after the third day of training. The peak amplitude (*15*) of I_A in response to 5.0-second positive step command pulses to −10 or 0 mV was significantly lower for the cells from paired animals than for cells from random or control animals (which had received no training) (Fig. 1 and Table 1). No significant differences were found for I_B. There were no significant differences between groups for "leak" current, holding current, or resting potential.

We have previously shown that I_A completely and rapidly becomes inactivated after a 5.0-second positive command pulse to 0 mV (*9, 12*). The time course of this exponential inactivation was measured here in two ways. In the first, we used a preceding pulse of variable duration to −30 mV followed by a test pulse to 0 mV. After 30 μsec, a test pulse alone was given. The ratio of peak I_A amplitude with a preceding pulse to peak I_A without a preceding pulse was calculated and plotted as a function of the duration of the preceding pulse. The half-time of decay of this curve, t_{pp}, was used as the measure of inactivation (*16*) rate of the preceding pulse potential. In the second method, we used twin, 5-second pulses to 0 mV with a 120 msec return to −60 mV between them. The ratio of peak I_A during the second pulse to the peak during the first was used as a measure of I_A inactivation.

These two measures were positively correlated with one another ($r_S = +.54$, $P < .02$). This twin pulse ratio for I_A was significantly lower for the paired cells than for random or control cells (Fig. 1 and Table 1). The half-time of decay (t_{pp}) for the paired cells was significantly lower only when compared with the control cells that were not significantly different from random cells (Table 1). All the significant differences (I_A peak amplitudes and inactivation ratios) were found for one of the three type B neurons in each eye, the medial cell (Fig. 1). The lateral and intermediate type B cells did not show differences with a small sample size and were not further analyzed here (*17*).

Since the peak amplitudes of I_A and the twin pulse ratios for random cells were not different from the naïve cells, the significant differences of I_A for paired cells are truly a result of the pairing of light with rotation rather than a direct result of phototransduction itself. This separation of phototransduction effects and conditioning effects on I_A and I_B was further demonstrated by an additional experiment. The I_A peak amplitude was unchanged during presentation of a light step, whereas I_B peak amplitude was markedly reduced (Fig. 1). The conditioning procedure, unlike light, changes I_A but leaves I_B unaltered.

The observed effect of repeated stimulus pairing on the type B photoreceptor during and immediately after acquisition of the associative learning behavior is prolonged membrane depolarization (*4, 18*). This prolonged depolarization probably arises from a synaptic enhancement of a light-induced, voltage-dependent Ca^{2+} current (*8*). Enhancement of this current is a direct consequence of paired

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stimulation of specific neural networks within and between the visual and statocyst pathways (*18*). This depolarization produced by stimulus pairing, therefore, encodes for brief periods (such as several hours) the occurrence of temporally associated stimuli. Prolonged membrane depolarization causes inactivation of I_A (*9*), and this inactivation is more persistent after longer depolarizing steps in association with light (*19*). Long-lasting reduction and an increased rate of inactivation of I_A as a consequence of prolonged depolarization encodes the occurrence of temporally associated stimuli on the days after the conditioning procedure.

Since I_A is a transient outward current, unlike the sustained light-induced Ca^{2+} current, it can have little direct effect on the steady-state membrane potential and thus, by itself, cannot cause depolarization. It can, however, determine the voltage response of the type B photoreceptor to light as well as the long-lasting depolarization that follows the light. At a resting membrane potential of −50 to −60 mV, there is a transient light-induced inward Na^+ current (Fig. 1D) but no sustained light-induced Ca^{2+} current (*8*). The sustained Ca^{2+} current occurs only at membrane potentials more positive than −30 mV (*8*). The magnitude of this Ca^{2+} current (and the prolonged depolarization with which it is associated) depends strictly, therefore, on the change of membrane potential resulting from the summation of two opposing transient currents: an outward I_A and an inward light-induced Na^+ current. If I_A has been reduced by stimulus pairing, I_{Na^+} will cause greater transient depolarization in response to light, and a larger voltage-dependent Ca^{2+} current will result in greater sustained depolarization during and after the light.

The probable origin of this change of I_A for the paired cells, prolonged depolarization in association with elevated intracellular Ca^{2+} (*19*), is apparently different from that of a transient K^+ current change that follows a single sensitizing stimulus to an *Aplysia* connective (*20*). This K^+ current was reduced without change of membrane potential (that is, during voltage clamp of the cell soma) in response to perfusion with serotonin and to electrical shock of the sensitizing connective (*20*). This difference in the origin of the K^+ current change as well as the nature of the K^+ current changed may help explain the known differences between two behavioral phenomena—sensitization and associative learning. As a consequence of the release of a neurochemical agent, the current changes of sensitization may have advantages for producing generalized behavioral effects such as arousal, which shows neither pairing nor stimulus specificity. As a consequence of depolarization that depends on the integrated response of a precisely ordered synaptic network to stimulus pairing, the change of I_A during associative learning may be better suited to preserve the information within specific stimulus relationships an organism encounters repeatedly within its environment (*8*).

Table 1. Voltage-dependent current characteristics of medial type B cells, given as means ± standard deviations (S.D.). Comparisons were made with *t*-tests. Abbreviations: I_A 60D, I_A elicited by command step to 0 mV; I_A 50D, I_A elicited by command step to −10 mV; I_{A_2}:I_{A_1}, ratio of maximum I_A value at 0 mV for the second to maximum value for the first of twin command pulses; t_{pp}, half-time of decay; *N*, number of medial cells, one per eye; P, paired; R, random; C, control; N.S., not significant.

Characteristic	Paired		Random		Paired control		Comparison	$P <$
	$\bar{X}$ ± S.D.	*N*	$\bar{X}$ ± S.D.	*N*	$\bar{X}$ ± S.D.	*N*		
I_A 60D (nA)	39.10 ± 10.70	10	55.13 ± 19.11	8	56.07 ± 15.27	7	P < R	.025
							P < C	.01
							R = C	N.S.
I_A 50D (nA)	16.82 ± 5.51	11	24.20 ± 9.53	7	24.92 ± 8.50	6	P < R	.05
							P < C	.025
							R = C	N.S.
I_{A_2}:I_{A_1}	0.348 ± 0.099	10	0.523 ± 0.110	8	0.439 ± 0.067	7	P < R	.005
							P < C	.001
							R = C	N.S.
t_{pp} (msec)	211.00 ± 18.17	5	250.00 ± 54.31	6	233 ± 13.51	5	P = R	N.S.
							P < C	.05
							R = C	N.S.

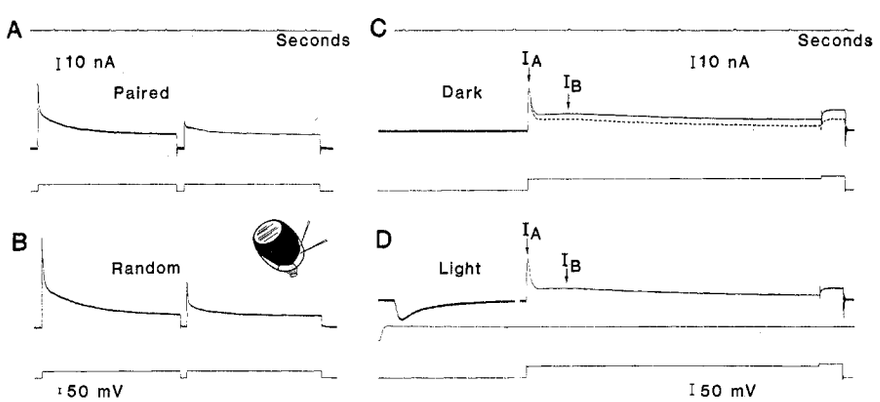

Fig. 1. Outward K^+ currents of type B cell. (A and B) Outward currents elicited by command pulses to 0 mV. Initial peaks are the early rapidly inactivating K^+ currents (I_A). Late outward K^+ currents (I_B) at 0 mV are only at threshold of the steep part of the activation curve and are thus obscured by the I_A currents elicited. I_B attains a maximum value approximately 1.0 second after the onset of the command pulse. Paired I_A is smaller than random I_A for both first and second command pulses. The ratio of paired I_A for second pulse to I_A for first pulse is smaller for paired than for random. Cartoon depicts impalement of medial type B cell with two microelectrodes. (C and D) Outward currents elicited by command pulses to −10 mV in darkness (C) and during light (D). The break in the current trace of (D) is a 10-second period until an approximate steady-state current during light was achieved. I_A is unchanged by light, whereas I_B is significantly reduced when measured 1.0 second after the onset of the command pulse to −10 mV and in response to a second command pulse to 0 mV 30 μsec after the first pulse. Dashed line in (C) represents superimposed current record of (D) to show differences. I_A is known to be entirely inactivated during the second command pulse.

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6. The word primary is used here to connote having a causal role in the associative learning since (i) the neural changes observed were intrinsic to this cell's soma membrane and (ii) this cell affects, through synaptic interactions, motor

neuronal activity responsible for actual behavior in response to light [(7); Y. Goh and D. L. Alkon, in preparation].
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13. The treatment condition of each animal was revealed after all voltage-clamp recordings had been analyzed and the quantitative measurements made.
14. A number of criteria for acceptability of impalement were used: (i) the resting potentials recorded by the voltage-recording and current passing electrodes should be approximately equal, (ii) the response to light recorded by each electrode should be approximately the same, (iii) the leak current at 0 mV should be ≤ 10 nA, (iv) the settling time of the voltage clamp should be ≤ 20 msec, and (v) the holding current at −60 mV should be ≤ 5 nA.
15. Corrections of I_A values for "leak" current were obtained by extrapolation from a linear portion of the current-voltage relation, which was generated with small positive and negative command pulses. I_A was also corrected for I_B, which was estimated from current values measured 1 second from the onset of the second of the twin pulses. This estimate agreed very closely with I_B values obtained with a test pulse (to 0 mV) occurring 30 μsec after a 5.0-second preliminary pulse to −10 mV. This correction for I_B was arrived at after numerous previous experiments (J. J. Shoukimas and D. L. Alkon, unpublished observations) that established I_B characteristics in the absence of I_A (after blocking with 4-aminopyridine).
16. The time constant for inactivation of I_A is much greater than the time constant of inactivation. Furthermore, there were no apparent between-group differences in the rising phase of I_A. Between-group differences in the decrement of I_A for the second of the twin pulses, therefore, were considered to largely reflect changes of inactivation of I_A rather than activation.
17. Other electrophysiological studies are also suggesting differences among the three type B cells with regard to changes during associative learning (T. Crow and D. L. Alkon, in preparation; J. Farley and D. L. Alkon, in preparation).
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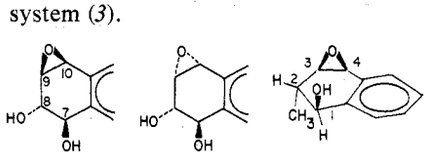
Internal Hydrogen Bond Formation in a *syn*-Hydroxyepoxide

Abstract. *The existence of an internal hydrogen bond in a compound representative of a* syn *diol epoxide (a possible intermediate in chemical carcinogenesis by certain polycyclic aromatic hydrocarbons) has been demonstrated by x-ray crystallographic and nuclear magnetic resonance studies. This internal hydrogen bond was found in 3,4-epoxy-2-methyl-1,2,3,4-tetrahydro-1-naphthol and was shown to persist in dioxane-water solutions containing up to 80 mole percent water. In this structure, the 1-hydroxy and 2-methyl groups are shown to occupy axial positions. In the* anti *diol epoxide, which has no internal hydrogen bond, analogous groups are equatorial. Crystals of the compound were unstable in the x-ray beam while the data were being collected (even at low temperatures), presumably as a result of decomposition.*

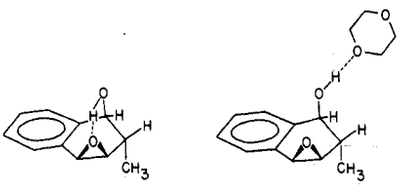
Initiation of carcinogenesis by benzo[*a*]pyrene (BaP) is considered by many to proceed by alkylation of DNA by an intermediate diol epoxide metabolite of BaP (*1*). Recent studies have shown that rat liver microsomes convert BaP, in part, to two diastereomeric diol epoxides [(−)-**1** and (+)-**2**] of high enantiomeric purity (*2*). The isomers are designated *syn* if the benzylic hydroxyl (the hydroxyl at position 7 of **1**) and epoxide groups project on the same side of the tetrahydrobenzene ring system (**1**), and *anti* if they lie on opposite sides (**2**). Isomer **2** has been identified as a potent carcinogen in newborn mice, and is thought to be the ultimate carcinogen of BaP in this system (*3*).



1 (*syn*) **2** (*anti*) **3** (*syn*)

Hulbert (*4*) pointed out that internal hydrogen bonding between the benzylic hydroxyl and epoxide groups is possible in *syn* diol epoxide **1**. Since this hydrogen bonding would weaken the C-O bonds of the epoxide, it was pointed out that nucleophilic attack at C(9) or C(10) of **1** would be facilitated. Indeed, the *syn* isomer (**1**) is about 160 times more reactive toward nucleophilic reaction at C(10) by the *p*-nitrothiophenolate ion in *t*-butyl alcohol than its *anti* isomer **2**, which appears not able to form such a stable intramolecular hydrogen bond (*5*). Presumably, then, the greater reactivity of the *syn* isomer causes it to interact with other macromolecules before it can reach the critical target for carcinogenesis. The structure of the *anti* BaP diol epoxide **2** has been studied by x-ray diffraction techniques (*6*). In addition, the probable three-dimensional structures of both isomers **1** and **2** have been constructed mathematically by merging known crystal structures (*7*).

We report here the crystal structure of a tetrahydronaphthalene epoxide **3** containing a benzylhydroxyl group located *syn* to the epoxide group (**3**). This compound is structurally related to the *syn* naphthalenediol epoxide (**1**), with the C(8) hydroxyl group of **1** replaced in **3** by a methyl group on C(2) (equivalent to C(8) of **1**). Compound **3** was prepared from *trans*-2-methyl-1-acetoxy-1,2,3,4-tetrahydronaphthalene by the following series of reactions: *N*-bromosuccinimide (NBS) bromination at C(4), dehydrobromination, and epoxidation to yield a mixture of the acetate of **3** and a diastereomeric acetate with the epoxide and acetate groups in a *trans* relation. The isomeric acetates were separated by alumina chromatography, and the *syn* acetate was hydrolyzed to **3**. Purification of **3** was effected by crystallization from an ether-pentane solution (*8*).



4 **5**

The ^1H nuclear magnetic resonance (NMR) spectrum of **3** in either CCl_4 or [$^2H_8$]dioxane revealed a small coupling of 2 Hz between H(1) and H(2), which suggests that the C(1) hydroxyl and C(2) methyl groups occupy axial positions. The ^1H NMR spectrum of **3** in CCl_4 showed an H(1)-OH coupling of 12 Hz

Table 1. Atomic parameters as fractions of cell edges with estimated standard deviation values in parentheses.

Atom	*x*	*y*	*z*	Atom	*x*	*y*	*z*
O(1)	0.4476(3)	0.7269(3)	−0.1283(4)	H(O1)	0.417(5)	0.647(5)	−0.051(7)
O(2)	0.4245(3)	0.6013(3)	0.2567(4)	H(C1)	0.679(3)	0.930(3)	−0.014(5)
C(1)	0.6138(4)	0.8298(4)	0.0350(6)	H(C2)	0.488(4)	0.888(3)	0.201(5)
C(2)	0.5727(4)	0.8758(4)	0.2448(6)	H(C3)	0.500(3)	0.771(3)	0.536(5)
C(3)	0.5247(4)	0.7542(4)	0.3805(6)	H(C4)	0.639(4)	0.607(4)	0.525(6)
C(4)	0.6229(4)	0.6575(4)	0.3922(6)	H(C5)	0.868(5)	0.558(5)	0.438(7)
C(5)	0.8891(4)	0.6194(4)	0.3145(6)	H(C6)	1.101(5)	0.592(4)	0.226(6)
C(6)	1.0161(5)	0.6420(4)	0.1948(8)	H(C7)	1.117(4)	0.756(4)	−0.073(6)
C(7)	1.0182(5)	0.7257(5)	0.0265(7)	H(C8)	0.892(4)	0.850(3)	−0.149(5)
C(8)	0.8902(5)	0.7887(4)	−0.0239(6)	H1(C11)	0.861(5)	1.006(5)	0.450(7)
C(9)	0.7608(4)	0.7669(4)	0.0936(6)	H2(C11)	0.765(4)	1.101(4)	0.314(5)
C(10)	0.7585(4)	0.6810(4)	0.2623(6)	H3(C11)	0.707(4)	1.029(3)	0.540(5)
C(11)	0.7295(5)	1.0175(4)	0.3933(7)				

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