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DECREASE OF GAP JUNCTION PERMEABILITY INDUCED BY DOPAMINE AND CYCLIC ADENOSINE 3′:5′-MONOPHOSPHATE IN HORIZONTAL CELLS OF TURTLE RETINA[1]

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Abstract

The axon terminals of the H1 horizontal cells of the turtle retina are electrically coupled by extensive gap junctions. Dopamine (10 nM to 10 μM) induces a narrowing of the receptive field profile of the H1 horizontal cell axon terminals, increases the coupling resistance between them, and decreases the diffusion of the dye Lucifer Yellow in the network formed by the coupled axon terminals. These actions of dopamine involve the activation of D1 receptors located on the membrane of the H1 horizontal cell axon terminals proper. Increases of the intracellular cyclic AMP concentration induced by either stimulating the adenylate cyclase activity with forskolin or inhibiting the phosphodiesterase activity with isobutylmethylxanthine, theophylline, aminophylline, or compound RO 20-1724 elicit effects similar to those of dopamine on the receptive field profile of the H1 horizontal cell axon terminals, on their coupling resistance, and on the diffusion of Lucifer Yellow in the axon terminal network. It is concluded that dopamine decreases the permeability of the gap junctions between the axon terminals of the H1 horizontal cells of the turtle retina and that this action probably involves cyclic AMP as a second messenger.

In contrast with chemical synapses, signals in electrotonic junctions are transmitted between the coupled neurons through gap junction channels, without intervention of neurotransmitters (see Bennett, 1977). However, it has been reported in recent years that electrical coupling between neurons could be altered by synaptic transmitters. In most of these cases the transmitter modified the electrical coupling by changing the conductance of the nonjunctional membrane (Spira and Bennett, 1972; Carew and Kandel, 1976; Spira et al., 1980). That a transmitter could induce a modification of the gap junction permeability itself has been recently suggested in two cases: the acinar cells of the pancreas (Findlay and Petersen, 1982) and the horizontal cells of the turtle retina (Piccolino et al., 1982). In the latter example, it was found that the GABA antagonists bicuculline and picrotoxin were able to decrease the permeability of the gap junctions connecting the axon terminals of the H1 horizontal cells of the turtle retina (H1ATs). The present paper reports another instance in which a neurotransmitter alters the permeability of the gap junctions of the turtle H1ATs.

The H1 horizontal cells of the turtle retina are highly differentiated spikeless cells which play the role of lateral associative interneurones at the outer plexiform layer. Morphologically, they are axon-bearing neurons showing a stereotyped image in Golgi preparations (Leeper, 1978a). They are endowed with a small soma, slender, radiating dendrites, and a thin axon of 0.2- to 0.5-μm diameter and about 100- to 400-μm length which ends in a stubby, thick terminal expansion. The cell bodies of the H1 horizontal cells are electrically coupled exclusively to other H1 somas (Byzov, 1975; Stewart, 1978; Piccolino et al., 1982) in a loose manner through small gap junctions (Witkovsky et al., 1983). On the other hand, the H1 axon terminals are electrically coupled only to other axon terminals (Byzov, 1975; Piccolino et al., 1982) through extensive gap junctions (Witkovsky et al., 1983). Therefore, the H1 cells of the turtle retina actually function as two different and electrically independent networks (see Gerschenfeld et al., 1982): the cell body network and the axon terminal network. Both H1 compartments give hyperpolarizing responses to light spot stimuli of every wavelength, but they differ in their receptive field properties (Simon, 1973; Piccolino et al., 1981). Small receptive field responses are recorded from the cell bodies and large receptive field responses from the H1ATs (Piccolino et al., 1982). Moreover, the two networks also differ in both their photoreceptor input (Leeper, 1978b; Leeper and Copenhagen, 1979) and in their feedback output on photoreceptors (see Piccolino and Neyton, 1982).

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Some years ago, Negishi and Drujan (1979) reported that dopamine (DA) evoked a narrowing of the receptive field profile of horizontal cells of a Cyprinid fish retina. The similarity of this effect with that found in our recent study on the action of GABA antagonists on the H1AT network of the turtle (Piccolino et al., 1982) led us to investigate the action of DA on this cell network. We have found that DA elicits a marked decrease of the summation area of the receptive field of the H1ATs, a fall in the electrical coupling between axon terminals, and a marked restriction of the diffusion of Lucifer Yellow dye in the network. It was also found that these effects of DA are all due to the activation of D1-type receptors to DA located on the H1AT membrane and are probably mediated by cyclic AMP as a second intracellular messenger.

Our results have been previously communicated in preliminary form (Gerschenfeld et al. 1982; Neyton et al., 1982). More recently, Teranishi et al. (1983) reported some similar results obtained in teleost fish retina.

Materials and Methods

The experiments were performed in perfused, moderately dark-adapted eyecup preparations of red-eared turtles (*Pseudemys scripta elegans*) that were obtained from Carolina Biological (Burlington, NC) and kept in aquaria. The animals were pithed, the eye was enucleated and hemisected, and the vitreous was carefully removed. This preparation was fixed to a chamber and continuously superfused with bicarbonate saline of the following composition (in millimolar concentration): NaCl, 110; KCl, 2.6; $NaHCO_3$, 22; $MgCl_2$, 2; $CaCl_2$, 2; and D-glucose, 10. This solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, thus keeping the pH at 7.4.

Intracellular recordings were made with glass microelectrodes drawn with a Brown-Flaming puller, filled with 4 M potassium acetate (resistance, 60 to 150 megohms). The recording pipettes were connected through DC amplifiers to a cathode ray oscilloscope, a Brush Gould pen recorder, and a magnetic tape recorder. The structures impaled were recognized as axon terminals of the H1 horizontal cells by their characteristic large hyperpolarizing responses and the extensive summation area of their receptive field (Simon, 1973; Piccolino et al. 1981).

Currents were injected intracellularly through the microelectrodes using a constant current source. In the intracellular injection experiments using two micropipettes, the cell penetrations were made using two mechanical microdrives; before penetrating the retina, the microelectrode tips were positioned over the vitreal surface of the retina under microscopic observation after momentarily stopping the saline flow.

The micropipettes used in the dye experiments, for both recording and injection, were filled with a solution of Lucifer Yellow of 30 gm/liter in 23 mM LiCl. They showed resistances of 300 to 600 megohms, and the injections were performed by applying through the microelectrodes 0.5-sec pulses of 5-nA hyperpolarizing current at 0.6 Hz for periods of 5 min. Ten to 30 min later the retina was stripped from the eyecup and fixed in a freshly prepared solution of formaldehyde at 40 gm/liter in 100 mM phosphate buffer (pH 7.2) for 10 to 12 hr at 4°C. The retina was dehydrated in graded alcohols, cleared in xylene, and whole mounted in Entellan. The microphotographs of these preparations were taken on Kodak Tri X-plan film in either a Zeiss or a Leitz fluorescence microscope provided with Neofluar optics.

Light stimulation of the retina preparations was provided by a double beam photostimulator. Light from two sources (tungsten iodide lamps) was passed via a conventional optic system through annular or circular apertures of different diameters. Neutral density filters were used to attenuate light intensity. The flux density of the unattenuated light on the retina was about 3×10^{-5} $\mu W/\mu m^2$. In some experiments a rectangular light stimulus was obtained by passing a light of one of the beams through a linear optical aperture whose length, width, and orientation could be continuously adjusted. The displacement of this stimulus was measured with a micrometer. Other details of stimulation are indicated in the legends of the figures.

Dopamine (3-hydroxytyramine), epinine, (±)-6,7-dihydroxy-2-aminotetralin (ADTN), apomorphine, and D-amphetamine were dissolved in the saline and protected from oxidation by adding 1 mM ascorbic acid. Haloperidol, clozapine, spiperone, domperidone, nomifensine, dihydroxynomifensine, sulpiride, and bicuculline were first dissolved in acid and then diluted in the saline. Isobutylmethylxanthine (IBMX), RO 20-1724, and picrotoxin were first dissolved in hot distilled water and then diluted in the saline. Forskolin was first dissolved in ethanol and then diluted in saline. The other drugs used were directly dissolved in the saline.

Results

Effects of dopamine and its agonists and antagonists on the receptive field properties of H1ATs. The *top recording* of Figure 1 gives an example of the typical pattern of the responses of the H1AT to white light stimuli of different configurations. The resting potential of the H1AT was −22 mV (in all of the experiments of this paper the dark potential of the H1AT in control conditions varied between −15 and −30 mV). When spots of light of varying diameters centered on the impaled element were flashed over the retina, they evoked hyperpolarizing responses which decreased in amplitude in parallel with the gradual decrease of the diameter of the spot. The maximal response was observed when the largest spot (5-mm diameter), which covered most of the surface of the eyecup preparation, was used (Fig. 1*a*). When the intensity of the light was maximal, such spot stimulation evoked responses of 42-mV amplitude for the cell illustrated. When anuli of light of 5-mm outer diameter and varying inner diameter were flashed, the responses increased in amplitude with the increase of the surface of the illuminated peripheral area (i.e., with the decrease in inner diameter; see Fig. 1*a*).

The application to the retina of a 5 μM concentration of DA for 20 min did not alter significantly the dark membrane potential of the H1AT and did not change its response to a 5-mm diameter light spot (Fig. 1*b*). Within a few minutes after the DA application the amplitude of the responses to spots of diameters between 2.2 and 0.25 mm became markedly increased, while the responses to the light anuli all decreased. Thus, DA elicited a narrowing of the receptive field profile of the H1AT. The same effects on the axon terminal responses were observed when DA was applied in the absence of ascorbic acid protection.

The DA action on the H1AT responses had a rapid onset (2 to 5 min) and was reversible, but only after prolonged washing with normal saline during 60 to 90 min, depending on the concentration of DA applied and the duration of application. In experiments in which different DA concentrations were

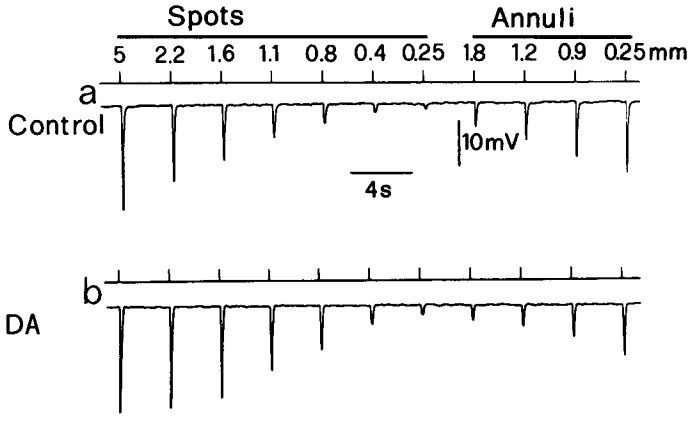

Figure 1. Effects of DA on the light responses of the H1ATs. Intracellular recordings of the responses of an H1AT to circular and anular light stimuli of different sizes. *a*, Control. *b*, After extracellular application of 5 μM DA for 20 min. In this and the following figures showing electrophysiological records, deflections in the trace above the recordings indicate the presentation of light stimuli (white light attenuated by 2.4 log units), and the numbers beside this trace indicate the diameter in millimeters of the light spots and the inner diameter of the anuli. The outer diameter of the anuli was always 5 mm.

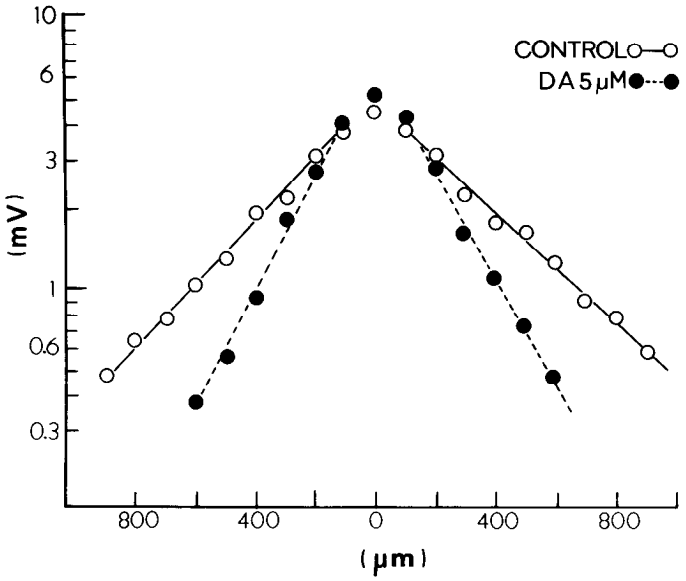

Figure 2. Effect of DA on the receptive field profile of the H1ATs. Peak amplitude of the responses of an H1AT to a light slit as a function of the deplacement of the slit across the receptive field, in the absence (*open circles*) and in the presence (*solid circles*) of 5 μM DA. The average of the responses to 10 light stimuli (in millivolts on the *ordinate*) is plotted against the distance from the slit to the H1AT receptive field center (in microns on the *abscissa*). The slit was 6 mm long and 150 μm wide, and the light was attenuated by 2.4 log units. The curves were fitted by eye.

assayed, the minimal concentration which elicited a significant modification of the H1AT receptive field was about 15 nM and the effect became maximal with 2 to 10 μM DA concentrations. In all of these cases and even at higher concentrations (up to 50 μM) assayed, DA neither significantly altered the H1AT dark potential nor produced a decrease of the maximal response to the larger spot stimuli.

A different way to assess the receptive field changes induced by DA is illustrated in the graph of Figure 2. In this experiment, a light slit of 6-mm length and 150-μm width was flashed on different positions on the H1AT receptive field. The amplitude of the average responses (10 stimulus presentations for each point), in a logarithmic scale on the ordinates, was plotted against the distance between the slit and the center of the receptive field. As first shown by Lamb (1976) for the turtle horizontal cells, the decay of the response amplitude with the displacement of the light slit is exponential; therefore, in a semilog plot, such as in Figure 2, it can be fitted by a straight line whose slope allows the measurement of the space constant (λ) of the H1AT network. From the graph of Figure 2, λ was calculated to be equal to 380 μm in normal conditions and 210 μm in the presence of 5 μM DA in the extracellular medium. Therefore, DA reduced the space constant of the network to 55% of its control value.

In 12 cells using the slit illumination procedure, the control values of λ ranged between 310 and 950 μm, but in all cases micromolar concentrations of DA reduced it by approximately 50% (mean ± SD, 50% ± 8%).

Modifications of the H1AT receptive field profile similar to those induced by DA were also observed when some well known DA agonists, such as epinine, ADTN, apomorphine, nomifensine, and dihydronomifensine (see Creese et al., 1982) were applied at concentrations varying between 200 nM and 50 μM. None of these agonists altered the dark membrane potential.

A series of DA antagonists (see Creese et al., 1982) was also assayed to determine their capability either to prevent or to reverse the DA effects on the receptive field properties of the H1ATs. Table I summarizes the results of such experiments. Flupenthixol, pifluthixol, fluphenazine, haloperidol, and (+)-butaclamol were able to prevent or block the DA effects, while the other antagonists had little or no blocking action. None of the effective antagonists produced a change in the dark potential. Such a pharmacological profile indicates that a DA receptor of the D1 type is involved in the action of DA on the H1AT network. Figures 3 and 4 illustrate two of these experiments. In Figure 3*a* the responses of an axon terminal to three light spots (diameters of 5, 1.6, and 1.1 mm) and to different light anuli (inner diameters of 1.8 and 1.2 mm) were recorded in normal conditions. DA (4 μM) was then applied to the retina, and the characteristic increase of the central responses and the

TABLE I

Efficacy of DA antagonists

This table expresses the qualitative results obtained in at least five cells for each compound. The efficacy was measured as the ability of the DA antagonist to prevent the narrowing of the H1AT receptive field profile induced by 5 μM DA.

Compound	Antagonist Action	Concentration Range
		μM
α-Flupenthixol	+++	1–50
α-Pifluthixol	+++	1–50
Haloperidol	+++	50–100
(+)-Butaclamol	+++	50–100
(−)-Butaclamol	−	100
Fluphenazine	+++	50–100
Clozapine	++	100
Spiperone	+	100
(+)-Domperidone	±	100
(−)-Sulpiride	−	120
(+)-Sulpiride	−	100

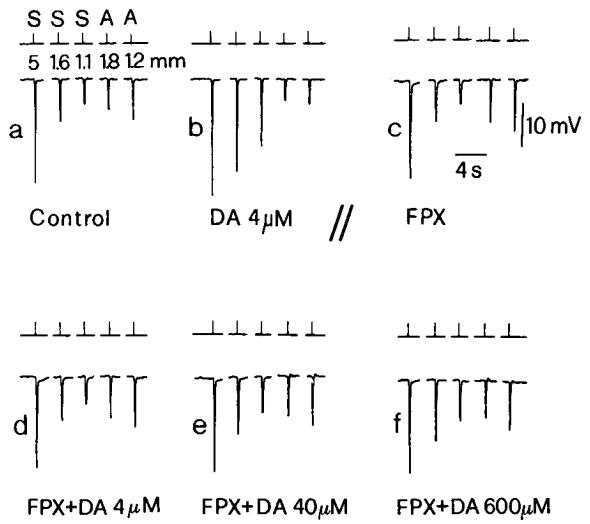

Figure 3. Effects of flupenthixol (*FPX*) on the DA-induced narrowing of the H1AT receptive field profile. *a*, Control responses of one H1AT to three different light spots (*S*) and two different anuli (*A*). *b*, Responses of the same H1AT to the same stimuli after application of 4 μM DA for 10 min. *c* to *f*, Responses to another H1AT that was impaled after removing DA by washing the preparation for 90 min. In *c*, the responses were obtained after a 40-min application of 50 μM flupenthixol. In *d*, *e*, and *f*, the same concentration of the antagonist was maintained in the extracellular space, and DA was added at increasing concentrations: 4 μM (*d*), 40 μM (*e*), 600 μM (*f*). Notice that in the presence of flupenthixol, the latter concentration did not attain the efficacy of 4 μM DA applied in the absence of the blocker.

reduction of the peripheral responses were observed after a few minutes (Fig. 3*b*). Between the recordings of Figure 3, *b* and *c*, the preparation was washed for more than 1 hr in normal saline, and a new H1AT was impaled. After confirming that the light responses had recovered their initial receptive field properties, 50 μM flupenthixol was applied for 40 min (Fig. 3*c*) keeping an H1AT impaled. At the end of this period it could be observed that the antagonist did not much alter the responses, with the exception of a small increase of the peripheral responses. When, as illustrated in Figure 3*d*, 4 μM DA was added to the flupenthixol-containing saline, no change in the responses was observed. Further increase of the DA concentration to 40 μM (Fig. 3*e*) and even to 600 μM (Fig. 3*f*) could only partially overcome the blocking effect of flupenthixol. From a comparison of the recordings of Figure 3, *b* and *f*, it can be seen that after flupenthixol treatment, a 150-fold increase in the DA concentration was not able to induce the same narrowing of the receptive profile attained by 4 μM DA in the absence of the antagonist. Flupenthixol and the other effective antagonists, at the concentrations used, took a rather long time (at least 30 min) to exert their blocking effects, and also a very prolonged washing out (4 hr or more) was necessary to reverse the blocking partially.

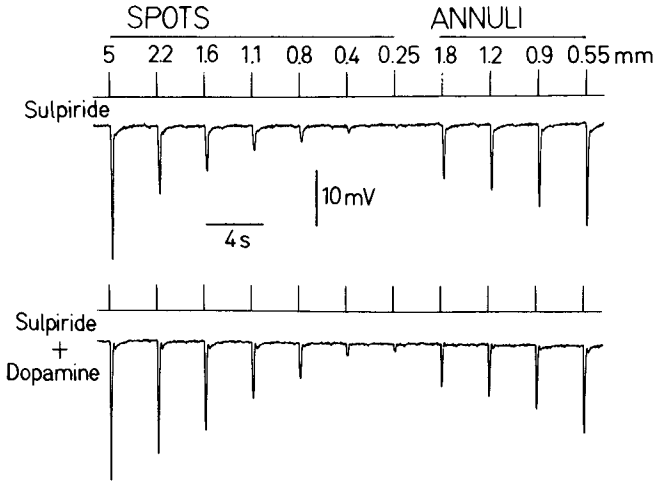

Figure 4. Lack of effect of (−)-sulpiride on the DA-induced narrowing of the H1AT receptive field profile. The H1AT light responses were all recorded after applying 120 μM sulpiride for more than 1 hr, first in the absence (*a*) and then in the presence (*b*) of 5 μM DA.

Figure 4 gives an example of the inefficacy of a D2 antagonist, sulpiride 120 μM, in preventing the action of DA.

Effect of DA on the spread of intracellularly injected current in the H1AT. Figure 5 illustrates the results obtained in one of seven experiments in which pulses of constant current were injected into one axon terminal and the resulting electrotonic potentials were recorded in another neighboring axon terminal. Before penetrating the retina, the microelectrode tips were positioned at a distance of less than 100 μm. In Figure 5, *a* and *b* correspond to control recordings from the two H1ATs bathed in a normal saline. The responses to a large spot, to a smaller spot, and to an anulus were first recorded, and then an inward current of 4 nA was injected into one of the axon terminals (Fig. 5*a*). In Figure 5, *c* and *d*, the same light and electrical stimuli were applied after a perfusion with a 5 μM concentration of DA for 10 min (notice the typical modifications of the light responses). In these conditions it was found that the amplitude of the electrotonic potential became increased (compare *b* and *d* in Fig. 5).

This result is similar to that obtained in our previous study on the action of GABA antagonists on the turtle H1AT network. Applying a similar analysis, we will try to show that, as in the case of the GABA antagonists, the effects of DA in Figure 5 result from a decrease in the coupling conductance between H1ATs.

For this purpose, we will refer mainly to a continuous model (Jack et al., 1975) previously used by Lamb (1976) to describe the electrical behavior of the H1AT network of the turtle. This model postulates that the electrical properties of a planar network of strongly coupled cells can be conveniently approx-

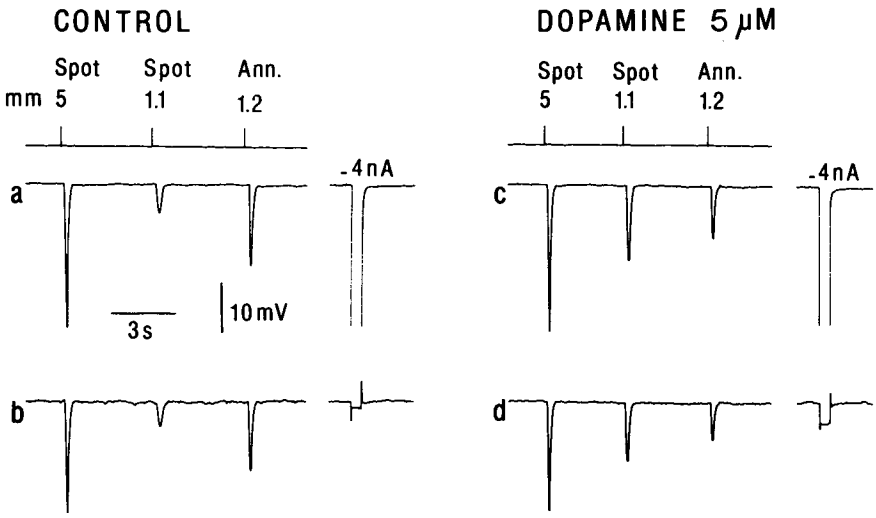

Figure 5. Effect of DA on the H1AT electrical coupling. The *upper* and *lower traces* correspond to two different H1ATs impaled simultaneously by independent microelectrodes whose tips were separated by less than 100 μm before penetrating the retina. *a* and *b*, Control recordings. After stimulation in sequence with two light spots and one anulus (*Ann.*), a 4-nA inward current pulse was injected into one of the H1ATs (*a*), and the resulting electrotonic potential was recorded in the other (*b*). *c* and *d*, Recordings obtained after application of 5 μM DA for 10 min. The same light stimuli were presented, and a current pulse of the same magnitude was injected, in *c*, in the same H1AT. Notice in *d* the increase in amplitude of the electrotonic potential.

imated, for many purposes, by those of a thin continuous sheet (a lamina) whose space constant λ is given by:

$$\lambda = (R_m/R_s)^{1/2} \quad (1)$$

in which R_m (ohms cm^2), the resistance of the lamina in the direction orthogonal to its planar extension ("membrane" leakage resistance), is the equivalent of the lumped extrajunctional membrane resistance of the cells, while R_s (ohms), the tangential "sheet" resistance, is the continuous equivalent of the coupling resistance of the network including the junctional and the cytoplasmic resistances. The voltage $V_i(r)$ evoked by a point injection of a current i at a distance r from the injection site is given, in the steady state situation by:

$$V_i(r) = (iR_s/2\pi)K_0(r/\lambda) \quad (2)$$

in which K_o is a modified Bessel function of zero order (Abramovitz and Stegun, 1972). Since K_o is a monotonically decreasing function of its argument, an increase in $V_i(r)$ when λ decreases (for constant r and i) cannot result from a simple decrease in R_m because R_m appears only implicitly through λ in the Bessel function argument. Thus, the observed increase of the coupling voltage in Figure 5*d* argues against a simple decrease of the extrajunctional membrane resistance of the H1AT network. In contrast, an increase of $V_i(r)$ as λ decreases could be due, *a priori*, only to an increase in R_s, since R_s appears also in the numerator of the factor multiplying K_o.

Actually, when studying the variations of V_ir), it can be seen that this increase of $V_i(r)$ when λ decreases as a result of an increase of R_s would effectively occur for certain values of r with respect to λ. By calculating the derivatives of $V_i(r)$ with respect to λ at fixed r and R_m, it can be shown that $V_i(r)$ presents a single maximum at $\lambda \simeq 0.6\ r$. Thus, $V_i(r)$ increases as λ decreases down to values 0.6 r and decreases for smaller values of λ. An illustration of this point is given by the family of curves plotted in Figure 1 of Detwiler and Hodgkin (1979). Since the distance between the electrode tips in the experiment of Figure 5 was less than 100 μm (thus, much shorter than the values of λ obtained in such experiments as the one illustrated in Fig. 2), the increase in the coupling potential induced by DA should necessarily involve an increase of the coupling resistance in the H1AT network.

Analogous conclusions can also be reached by applying discrete models of the H1AT. In fact, as was elegantly shown by Detwiler and Hodgkin (1979), the predicted values of $V_i(r)$ in such discrete models are similar to those of the lamina, provided that the cells (or elements) are closely coupled; that is, if the network space constant is larger than the cell spacing (distance between adjacent cell centers). This condition is satisfied by the H1AT network, whose space constant generally is 10 times larger than the average cell spacing (see Witkovsky et al., 1983, and the legend of Fig. 6). At this point, it should be noted that the continuous model cannot be used to predict the value of the coupling potential in the cell in which the current is injected. In equation 2, $V_i(r) \to \infty$ when $r \to 0$, and that has no physiological meaning. To predict what happens in the cell in which the current is injected, it is necessary to use a discrete model of the H1AT network (see below and legend of Fig. 6.)

A graphical illustration of the predictions of the models mentioned above is shown in Figure 6 in which the spatial decay of $V(r)$ following a 1-nA injection in a point of the H1AT network is calculated in three different hypothetical conditions. The *open circles curve* corresponds to the values of $V(r)$ in "control" conditions in which λ was assumed to be 450 μm; the *triangles curve* corresponds to a condition in which λ was decreased to half by a decrease in R_m; and the *solid circles curve* corresponds to a similar decrease of λ due to an increase in R_s. For the nonzero values of r, the three curves were calculated

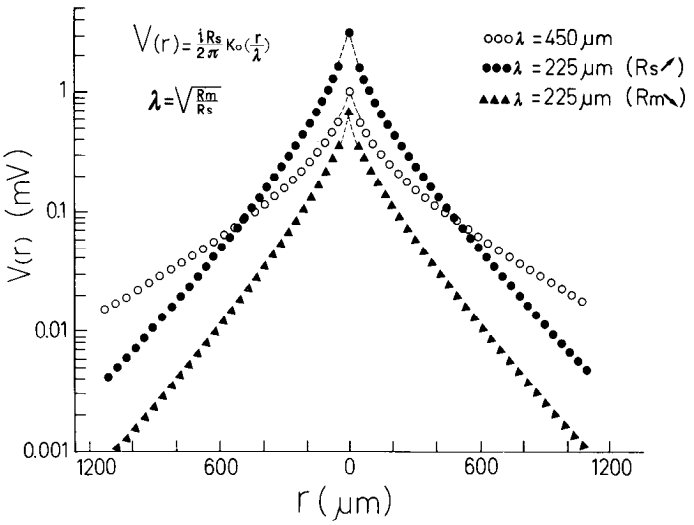

Figure 6. Spatial spread of current in an electrical network modeling the H1AT network. The voltage drop $V(r)$ evoked by injecting a 1-nA outward current pulse in a point of the network is plotted on the *ordinate* against the distance from the injection point on the *abscissa*. As indicated, the network space constant λ = 450 μM for the *open circles curve* ("control" curve), and it is assumed to be reduced to 225 μM by either a decrease in R_m, the "membrane" leakage resistance (*triangles curve*), or an increase of R_s, the equivalent of the coupling resistance (*solid circles curve*). As mentioned in the text, the values of $V(r)$ for $r \neq 0$ were obtained from the equation at the *top left* of the figure. The value of R_s necessary to fit the control curve was calculated from equation 15 in the paper of Lamb and Simon (1976), which corresponds to a discrete network model (the "square grid" model). For a network of strongly coupled cells, i.e., when λ is larger than the cell spacing (which is the case for the H1AT network), that equation can be simplified to the following:

$$R_s = \frac{V_0}{i}\frac{\pi}{2}\frac{D^2 + 4\lambda^2}{\lambda^2}\frac{1}{K\left(\left[\frac{4\lambda^2}{D^2 + 4\lambda^2}\right]^2\right)}$$

in which V_o is the voltage in the central element of the network in which the current i is injected, D is the cell spacing, and $K(x)$ is the complete elliptic integral of the first kind (Abramovitz and Stegun, 1972). For the purpose of calculation, V_o/i was assumed to be 1 megohm, which is in the range of our experimental measurements of the input resistance of H1ATs in control conditions (see Baylor et al., 1971). D was calculated to be 45 μm from counting the H1AT density in our Lucifer Yellow experiments (see also Witkovsky et al., 1983). The "square grid" model was also used to calculate the value of V_o for both the *solid circles curve* and the *triangles curve*.

using equation 2. The value of R_s in the control curve was calculated by reference to a discrete network model (the "square grid" of Lamb and Simon, 1976) which also allowed us to extend the analysis to the central element of the network ($r = 0$). The details of these calculations are briefly described in the legend of Figure 6. The curves of Figure 6 indicate that only when R_s is increased, increased values of $V(r)$ are observed at values of $r < 500$ μm. In contrast, when R_m is decreased, $V(r)$ is smaller than its control value for all of the values of r.

In conclusion, the current injection experiments indicate that DA induces a modification in the spread of current in the H1AT network due to a increase in the coupling resistance between axon terminals.

Effects of DA on the diffusion of Lucifer Yellow dye in the H1AT network. The dye Lucifer Yellow permeates gap junction channels (Stewart, 1981) and this was observed in particular in the case of electrically coupled horizontal cells of different vertebrate retinas (see Dacheux and Raviola, 1982), including

the H1AT network of the turtle (Stewart, 1978; Piccolino et al., 1982).

Figure 7*a* illustrates the typical pattern of diffusion of Lucifer Yellow after intracellular injection into one H1AT of turtle retina bathed in normal saline. The most fluorescent structure appearing in the microphotograph is the injected axon terminal from which the dye diffused to a rich network of interconnected H1ATs. A certain number of cell bodies corresponding to the stained terminals were filled by back diffusion of the dye through the slender axon fibers.

In 12 dye injection experiments in which DA (5 to 50 μM) was applied, the pictures obtained were similar to that of Figure 7*c*. In this case the dye was injected after a 10-min application of 10 μM DA, and it can be seen that the diffusion of the dye became restricted to very few H1ATs beside the injected one. The cell body corresponding to the injected axon was intensely stained by backfilling, thus showing that the lack of staining of the network was not due to an impairment in the dye diffusion in the cell cytoplasm.

As for the action on the receptive field profile, the effects of DA on the diffusion of Lucifer Yellow in the H1AT network could be prevented by an application of flupenthixol.

Effects of DA after suppression of synaptic transmission in the retina by a Co^{2+}-containing medium. In the experiments described up to here, we have applied DA to the whole retina and recorded its effects on the H1AT network. The question which arises immediately is whether the modifications induced by DA on the coupling between H1ATs in the network are due to the activation of DA receptors located in the axon terminal membrane proper or are the result of an indirect action of DA via other neurons somehow connected to the H1ATs. To clarify this problem, a series of experiments was performed in which we attempted to disconnect the H1ATs from their chemical synaptic inputs by introducing Co^{2+} ions in the extracellular medium to block synaptic transmission (Cervetto and Piccolino, 1974). When the retina was bathed in a saline to which 3 to 4 mM Co^{2+} was added, the H1ATs became hyperpolarized by 30 to 50 mV, and their light responses were blocked as a consequence of the suppression of the transmitter release from the photoreceptors. In such conditions, the recording of the effects of DA on the receptive field profile of the H1ATs became impossible. We tested then the effect of DA on the Lucifer Yellow diffusion in the network.

Figure 7, *e* and *f*, illustrates the effects of Co^{2+} alone and Co^{2+} plus DA on the dye diffusion in the H1AT network. In seven experiments, the addition of 3 mM Co^{2+} to the extracellular medium induced a mild, but consistent, diminution of the diffusion of Lucifer Yellow in the H1AT network (compare *e* and *a* of Fig. 7). In 10 Co^{2+}-treated retinas, 10 μM DA was applied after observing the Co^{2+}-induced hyperpolarization block of the H1AT light responses. In these conditions, as in control saline, DA was still able to reduce the dye diffusion in the network (Fig. 7*f*), and it had no effect on the H1AT membrane potential. Thus, the amine exerted its action even in the absence of any synaptic transmitter release.

Effects of pharmacological agents that produce an increase of intracellular cyclic AMP. We have shown above that DA exerted its effects by activating D1-type receptors. These receptors have been shown in both brain and retina (Brown and Makman, 1972; Kebabian et al., 1972; Watling and Dowling, 1981) to be linked to the activation of adenylate cyclase, the enzyme which synthesizes cyclic AMP, a second messenger of both hormones (see Sutherland, 1972) and chemical transmitters (see Nathanson, 1977). It was then natural to explore whether pharmacological agents known to induce an increase in intracellular cyclic AMP were able to evoke similar effects to those of DA and its agonists.

The first procedure used to increase the intracellular cyclic AMP concentration was to stimulate its synthesis by applying forskolin, a diterpene compound known to stimulate the activity of adenylate cyclase in many intact and broken cell preparations (Seamon et al., 1981), independently from the activation of DA receptors.

Figure 8 gives an example of the results obtained in eight experiments in which forskolin was applied to the retina. As in the case of Figure 5, two independent microelectrodes were used, and before penetrating the retina their tips were separated by less than 100 μm. Two H1AT recordings were then obtained. The responses to two light spots (diameters of 0.5 and 1.6 mm) and to a light anulus (inner diameter of 0.9 mm) were first recorded in the retina bathed in normal saline; 1-nA current pulses of both polarities were then injected through one of the microelectrodes (Fig. 8*a*), and the resulting electrotonic potentials were recorded with the other (Fig. 3*b*). The application of 5 μM forskolin produced, as in the case of DA, an increase in the amplitude of the response to the smaller central spot and a decrease of the peripheral response (Fig. 8, *c* and *d*). When the same current pulses were injected in the presence of forskolin, much larger electrotonic potentials were recorded (Fig. 8*d*). These results are similar to those obtained with DA, thus suggesting that forskolin increases the coupling resistance in the H1AT network.

Figure 7*b* shows that, beside its electrophysiological effects, forskolin also restricted the diffusion of the intracellularly injected dye Lucifer Yellow to a few H1ATs around the injected axon terminal and to its backfilled cell body. None of the effects of forskolin was observed when a similar concentration of its vehicle (alcohol 0.05%) was applied alone. Moreover, the only difference found between the actions of DA and forskolin was that the DA antagonists did not alter the forskolin effects.

The second procedure used to increase the cyclic AMP concentration was to inhibit the cyclic nucleotide phosphodiesterase (PDE), the enzyme which inactivates cyclic AMP. For this purpose we used some well known inhibitors such as IBMX, theophylline, aminophylline, and the nonxanthinic compound RO 20-1724. Figure 9 illustrates one of a series of five experiments in which two different H1ATs were impaled with two independent microelectrodes whose tips were set before the retina penetration at a distance smaller than 100 μm. After impaling the H1ATs and recording their responses to two light spots (diameters of 5 and 1.1 mm) and to an anulus (inner diameter of 0.9 mm), inward current pulses were alternatively injected in each axon while recording the resulting coupling potentials in the other (Fig. 9, *a* and *b*). After 10 min of application of 200 μM IBMX to the retina (Fig. 9, *b* and *d*), the responses to the smaller light spot were markedly increased, while the amplitude of the peripheral responses was diminished. Moreover all of the light responses became much slower, in particular in their decaying phase, and the membrane potential became depolarized by about 7 mV. As in the case of DA and forskolin, the electrotonic potentials increased in amplitude under the effect of IBMX, thus indicating a similar increase in the network coupling resistance. Similar effects could also been recorded using lower IBMX concentrations (down to 20 μM).

IBMX also mimicked the effects of both DA and forskolin on the diffusion of Lucifer Yellow in the H1ATs. Figure 7*d* shows that the application of 50 μM IBMX to the retina also caused a marked limitation of the diffusion of Lucifer Yellow, the fluorescent staining being reduced to few H1ATs and to the backfilling of the soma corresponding to the injected terminal.

The other assayed PDE inhibitors elicited modifications of the H1AT receptive field profile similar to those produced by IBMX, but with even higher concentrations (0.5 to 2 mM) the effects were less pronounced. These PDE inhibitors were also found to slow down the H1AT responses.

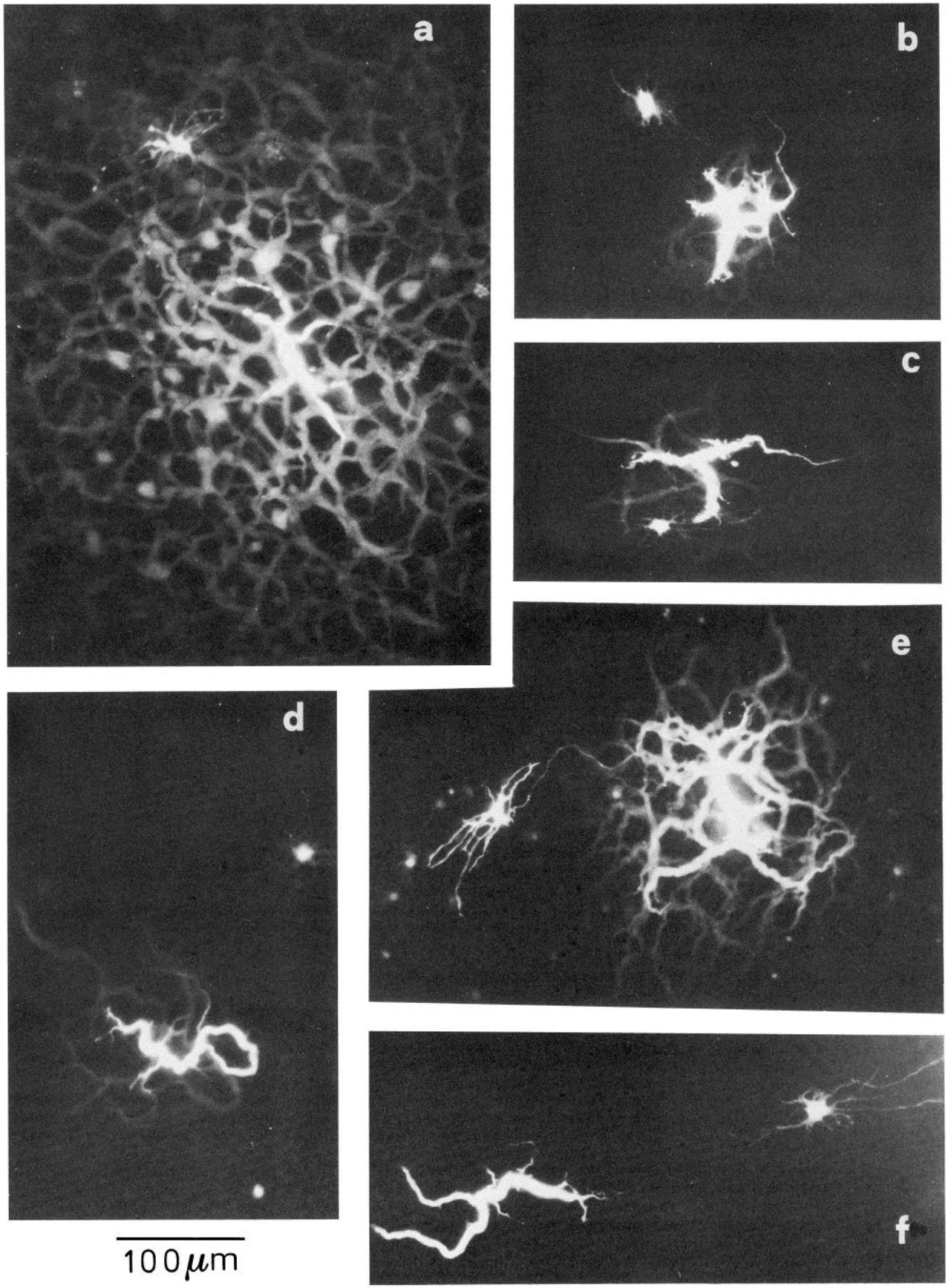

Figure 7. Diffusion of intracellularly injected Lucifer Yellow in the H1AT network. *a*, Control. Notice the extension of the dye diffusion and the backfilling of numerous H1 cell bodies, and among them, the stellate perikaryon at the *top left* which probably corresponds to the injected H1AT. *b*, After bath application of 10 μM forskolin. *c*, In the presence of 10 μM DA in the extracellular medium. *d*, After application of 50 μM IBMX. *e*, In the presence of 3 mM cobalt chloride in the bath. The injection of the dye was performed after hyperpolarization and block of the H1AT light responses. *f*, In the continuous presence of cobalt, 10 μM DA was added to the extracellular medium.

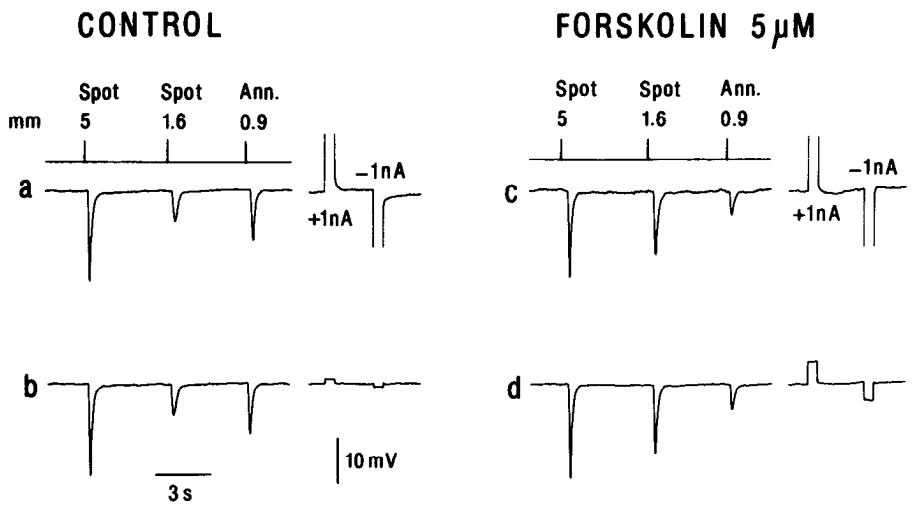

Figure 8. Effects of forskolin on the H1AT light responses and coupling potentials. Intracellular recordings were obtained simultaneously from two H1ATs (one of them recorded in the *upper traces* and the other in the *lower traces*). The distance between the microelectrode tips was 100 μm before the retina penetration. *a* and *b*, Light responses and intracellular current injection in control conditions. *c* and *d*, Application of the same light and electrical stimuli in the presence of 5 μM forskolin in the saline.

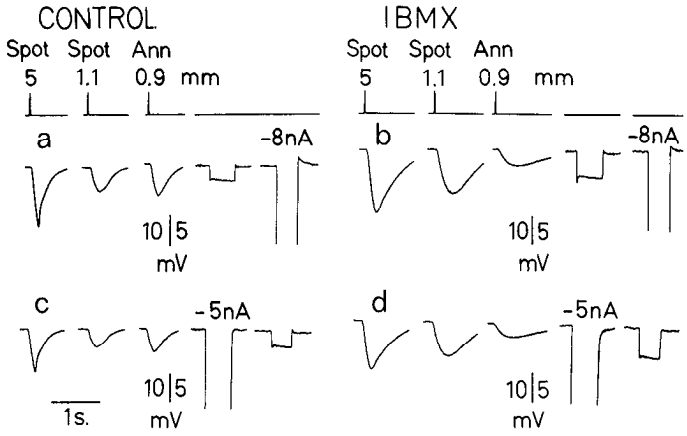

Figure 9. Effects of IBMX on the light responses and current spread in the H1AT network. The *upper* and the *lower recordings* were obtained from two different H1ATs impaled by two microelectrodes set at less than 100 μm before entering the retina. *a* and *c*, Recordings of responses to light and to intracellular current pulse injection in normal saline. *b* and *d*, The same stimuli were applied in the presence of 200 μM IBMX. Notice the depolarizaton of the H1ATs, the slowing down of the light-evoked responses, and the increase in the electrotonic potentials induced by IBMX.

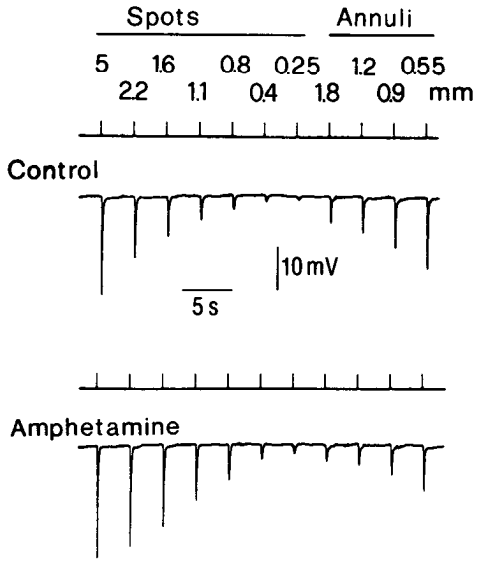

Figure 10. Effects of amphetamine on the H1AT light responses. *Top trace*, Control responses of a H1AT to light stimuli. *Bottom trace*, Responses to the same light stimuli after application of 40 μM D-amphetamine.

The depolarization of the H1ATs and the slowing down of the axon terminal light responses by the PDE inhibitors were probably a reflection of events taking place in the red cones. We could observe in three experiments on red cones that they also were depolarized and their responses similarly slowed down by the PDE inhibitors (not shown). Similar observations were previously made in *Bufo marinus* rods (Capovilla et al. 1982).

Amphetamine experiments. Since DA showed so striking effects on the H1AT network, it was worth exploring whether DA could be released in the retina and whether this endogenous release could affect the H1AT network. For this purpose we investigated the effects of D-amphetamine, a drug known to have the property of releasing catecholamines from synaptic varicosities and terminals (see Raiteri and Levi, 1978), expecting that its application to the retina will mimic the effects of DA.

Indeed, Figure 10 shows (as in the other 15 experiments) that 40 μM D-amphetamine evoked a similar narrowing of the receptive field profile of the H1ATs as DA. When short applications of the drug were repeated, it was often observed that after the maximal effect of the first application, the following D-amphetamine treatments became less and less effective.

An indication that D-amphetamine probably acted by releasing DA was given by the observation that a DA receptor antagonist, such as flupenthixol (50 μM) was able to prevent the effects of D-amphetamine on the H1ATs.

Experiments combining the application of a DA and a GABA antagonist. As we previously reported (Piccolino et al., 1982),

GABA antagonists, such as bicuculline and picrotoxin, exert the same effects as DA on the H1AT properties. It was then interesting to investigate whether these two kinds of pharmacological agents interacted.

Figure 11 shows an experiment of a series of five performed with such purpose. The receptive field properties of an axon terminal were studied using a series of light spots and anuli of varying configurations (Fig. 11, *top*). Flupenthixol (50 μM) was first applied for more than 40 min. Thereafter, when the DA receptors were likely blocked, 50 μM bicuculline was added to the flupenthixol-containing medium and applied for more than 20 min. In control conditions, such a bicuculline concentration should have induced the expected change in the H1AT receptive field properties within 5 to 10 min. However, no effect of bicuculline on the axon terminal light responses was observed after flupenthixol treatment (compare Fig. 11, *middle* and *bottom*). Other DA antagonists which were able to block the DA effects on the H1AT network equally prevented the effects of bicuculline.

Discussion

The main finding of the present work is that DA induces a narrowing of the receptive field profile of the turtle H1ATs due to a decrease of the permeability of the gap junctions in the network. This effect of DA involves the activation of D1 receptors on the H1AT membrane and is probably mediated by cyclic AMP.

DA decreases the gap junction conductance. With the exception of a small number of known gap junctions (see, for instance, Audesirk et al., 1982), an excellent unequivocal relation has been reported to exist between the presence of electrical coupling via gap junctions and the intercellular diffusion of Lucifer Yellow (Stewart, 1981). In particular, the decrease in the passage of dye between coupled cells was always associated with a decrease of the permeability of the gap junction channels (Spray et al., 1979; Murphy et al., 1983). In our experiments, if the decrease of the electrical coupling induced by DA were due to a pure decrease of the extrajunctional membrane resistance of the H1ATs, no changes in the dye diffusion should have been observed, because the dye does not normally permeate the plasma membrane (Bennett et al., 1978; Stewart, 1981). Thus, the decrease of dye diffusion in our experiments strongly indicates that DA decreases the permeability of the gap junctions between H1ATs.

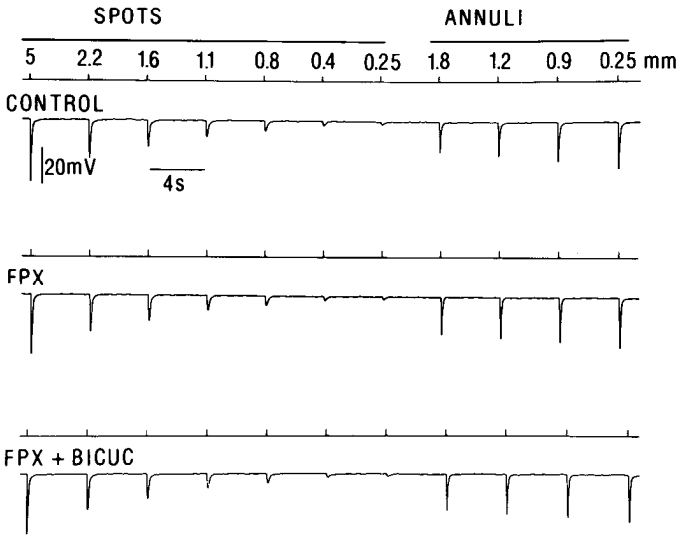

Figure 11. Experiments combining a DA antagonist and a GABA antagonist. *Top trace*, Control responses of an H1AT to different light stimuli. *Middle trace*, Responses of the same H1AT after application of 50 μM flupenthixol (*FPX*) for 45 min. *Bottom trace*, Recording obtained 20 min after adding 50 μM bicuculline (*BICUC*) to the flupenthixol-containing medium.

A possible objection to the weight of the Lucifer Yellow diffusion argument could be that DA induced a reduction of the permeability of the gap junction to the dye but not to ions carrying the currents between H1ATs. That such is not the case is shown by the results of the current injection experiments which indicate that DA decreases the network coupling conductance.

Although the effects of DA on the H1AT electrical coupling cannot be accounted for by an alteration of the extrajunctional membrane resistance, they cannot exclude it. However, both in the presence and the absence of Co^{2+} in the extracellular medium, i.e., when the membrane potential is either at the dark or at the light level, and when the membrane conductance is either affected or not by the photoreceptor transmitter/s, DA did not change the membrane potential. These observations suggest that DA does not alter the extrajunctional membrane conductance.

DA receptors are located on the H1ATs. The DA-induced decrease in the conductance of the gap junctions between H1ATs is, therefore, the expression of a novel modulatory mechanism in which the permeability of intercellular channels connecting the cytoplasm of the cells is modulated by a chemical transmitter that would act on receptors located on the outer surface of their plasma membrane. The persistence of the DA effects on the dye diffusion after suppressing completely the synaptic activity in the retina with Co^{2+} ions is a strong indication that the DA receptors are actually located on the H1AT membrane.

However, it can be argued that Co^{2+} ions only suppress the Ca^{2+}-dependent transmitter release but that they should not affect a Ca^{2+}-independent transmitter release as the one observed in the toad horizontal cells (Schwartz, 1982). Nevertheless, it must be remembered that this Ca^{2+}-independent release depends on a depolarization of the membrane. Since the turtle H1ATs are strongly hyperpolarized by Co^{2+} ions (Cervetto and Piccolino, 1974), the Ca^{2+}-independent release of transmitter should also be suppressed by the application of Co^{2+} ions. Therefore, the DA receptors should be located on the H1AT membrane proper.

Possible involvement of cyclic AMP as a second messenger of DA. Since the available evidence (see Loewenstein, 1981; Spray et al., 1984) indicates that the permeability of the gap junction can be altered by changes in the intracellular milieu, it was probable that the action of DA involved an intracellular second messenger. The evidence presented in this paper suggests that cyclic AMP could be this second messenger.

A first indication of the intervention of a second messenger was the fact that, after short applications of micromolar DA concentrations, the time to reverse its effects was too prolonged to correspond to a simple washout from the receptors exposed to the extracellular space. Moreover, our pharmacological experiments clearly indicate that the effects of DA on the H1ATs involve the activation of D1-type receptors to the amine. This type of DA receptors has been shown to be involved in the activation of the enzyme adenylate cyclase in both the brain (Kebabian et al., 1972) and the retina (Brown and Makman, 1972; Watling and Dowling, 1981). More recently, DA has been reported to stimulate the adenylate cyclase activity in isolated horizontal cells of fish retina (Van Buskirk and Dowling, 1981).

Our experiments are much in favor of an involvement of cyclic AMP in the mediation of the DA-induced decrease of the gap junction conductance. Indeed, pharmacological agents which increase the intracellular concentration of cyclic AMP independently from a DA receptor activation, either by stimu-

lating the adenylate cyclase activity or by inhibiting the PDE activity, mimic DA effects on the receptive field properties, the spread of current and the diffusion of Lucifer Yellow in the H1AT network.

We ignore at present how the cyclic AMP increase could lead to the closing of the gap junction channels. It has been demonstrated in recent years that either an increase in the intracellular calcium concentration (Rose and Loewenstein, 1976) or an acidification of the intracellular milieu (Turin and Warner, 1977; Giaume et al., 1980; Spray et al., 1981) cause cell uncoupling by increasing the junctional resistance, but we do not know if the increase in intracellular cyclic AMP modifies the intracellular Ca^{2+} or pH in the H1ATs or if cAMP could intervene by phosphorylating a protein involved in the gap junction permeability.

Has the DA-induced H1AT uncoupling a functional significance? The ensemble of the experimental evidence discussed above would be in favor of a possible participation of DA in a modulation of the electrical coupling in the turtle H1AT network in physiological conditions. If such was the case, one might expect to find a dopamine-containing compartment from which DA could be released at the outer plexiform layer of the turtle retina. A dopaminergic neuron, the interplexiform cell, establishing contacts with horizontal cells at the outer plexiform layer, has been observed in teleost fish (Ehinger et al., 1969), New World monkeys (Dowling and Ehinger, 1975), and humans (Frederick et al., 1982), and it also exists in the rat retina (Nguyen-Legros et al., 1981). Such a type of cell has not yet been identified in the turtle retina. Catecholaminergic amacrine cells have been observed in this retina using both the Falck-Hillarp and immunohistochemical techniques, but no evidence has been found of an extension of their dendritic processes toward the distal retina (Witkovsky et al., 1984). In spite of this lack of histological evidence, our experiments of amphetamine application to the turtle retina suggest the existence of a dopamine-releasing compartment near the L-horizontal cells, since this drug, known to release catecholamines (see Raiteri and Levi, 1978), mimicked the effects of DA on the receptive field profile of the H1AT network, and these effects were prevented by DA antagonists.

Another aspect which deserves discussion concerns the influence of light on the retinal DA. From recent reports on the effects of light on the synthesis (Da Prada, 1977; Iuvone et al., 1980), uptake, storage, release (Kramer, 1971), and metabolism of DA (Parkinson and Rando, 1983), as well as on the sensitivity of the DA receptors (Spano et al., 1977), it appears that the retinal DA system is activated by light. If the dopaminergic neurons that would intervene in the modulation of the coupling of the H1ATs would also be activated by light, then DA could intervene in a light-activated modulation of the coupling properties of the H1AT network. Consistent with this view, one of us has recently observed (M. Piccolino, unpublished data) a narrowing of the H1AT receptive field in the presence of diffuse background light.

A DA-GABA interaction in the turtle retina? Another question related to the possible physiological role of DA in the turtle outer plexiform layer arises from the parallelism between the effects of both DA and the GABA antagonists already mentioned. Our experiments in which bicuculline was applied in the presence of flupenthixol were addressed to understand whether the similar effects of both DA and GABA antagonists on the H1AT network could be somehow related. If bicuculline and picrotoxin act on GABA receptors also located in the H1AT membrane by a mechanism independent from DA, their effect should persist when the DA receptors are blocked by DA antagonists. Our experiments demonstrate that this is not the case because GABA antagonists failed to alter the H1AT receptive field in the presence of DA antagonists. One possibility is that the action of the GABA antagonist is not directly exerted on the H1AT membrane and that the GABA receptors are located on a cell different from the H1 horizontal cell, for instance, on a dopaminergic cell involved in the H1AT coupling modulation.

In view of the evidence indicating that some of the horizontal cells of many species of lower vertebrates (Marc et al., 1978; Holleyfield et al., 1979; Lam et al., 1979; Schwartz, 1982), including the H1 cells of the turtle (W. Eldred, H. J. Karten, and D. Oertel, personal communication), are GABAergic, one may postulate that the H1AT and the dopaminergic cell influencing its receptive field properties constitute a loop circuit, in which GABA released from the H1AT would depress or stop the release of DA from the dopaminergic cell. The application of GABA antagonists would then block the depressing action of GABA on the DA release from the dopaminergic cell and, thus, facilitate the physiological release of DA. Therefore, the final common pathway of the pharmacological actions of both DA and the GABA antagonists which lead to the fall in gap junction permeability in the H1AT network would be the increase in intracellular cyclic AMP through the activation of adenylate cyclase, via the D1 receptors to DA.

Recent biochemical experiments on rat retina, in which a dopaminergic interplexiform cell has been described, suggest a similar DA-GABA interaction (Kamp and Morgan, 1980, Morgan and Kamp, 1980). It was found both in the intact retina and in cell suspension preparations that GABA antagonists increased the release of DA and that GABA agonists suppressed it.

In the retinas of teleost fish, effects of DA on the receptive field properties (Laufer et al., 1981), the electrical coupling (Laufer, 1982), and the diffusion of Lucifer Yellow between horizontal cells have been reported to be similar to those reported in this paper (Teranishi et al., 1983). The later authors reported recently that some DA antagonists blocked the effects of DA on the receptive field properties and that IBMX mimicks the effects of DA. However, Yazulla and Kleinschmidt (1982) have found by autoradiographic methods that DA reduces the release of GABA from teleost horizontal cells and that such action appears associated with an increase in cAMP concentration. A possible interpretation of all of these results taken together could be that DA evokes the uncoupling between horizontal cells indirectly through the GABA system. In the turtle retina, however, if GABA would mediate the effects of DA, the action of bicuculline should not be blocked by flupenthixol. An alternative explanation of the results from the teleost fish retina would be that DA, by increasing cAMP, could exert its uncoupling effect and, at the same time, reduce the release of GABA from the horizontal cells. This action of DA on the GABA release would reinforce its direct effect on the coupling if one assumes, as we already proposed, that GABA affects the horizontal cell coupling by decreasing the DA release.

Finally, whatever would be the functional implications of the effects of DA on the H1AT coupling, it is important to retain as the main conclusion of the present work the demonstration that a transmitter is able to regulate the permeability of gap junctions.

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