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The glutamate analog 2-amino-4-phosphonobutyrate antagonizes synaptic transmission from cones to horizontal cells in the goldfish retina

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ABSTRACT **In the retina, the glutamate analog 2-amino-4-phosphonobutyrate (APB) distinguishes a class of glutamate receptors that is thought to be found only on depolarizing bipolar cells (DBCs). We now report that APB is a potent antagonist of cone-driven horizontal cells in the goldfish retina. APB hyperpolarized the membrane to the same potential as cobalt Ringer's and blocked the light responses. APB acted specifically on the cone pathway, as it had no effect on rod-driven horizontal cells. The lowest effective APB concentration for antagonistic action on the horizontal cells (≈2 μM) was similar to the concentration for agonist action on DBCs. APB was not able to block the actions of exogenous glutamate or kainate on horizontal cells. We propose that the action of APB on the cone–horizontal cell synapse is mediated at a site that is distinct from the glutamate and kainate binding site. Therefore, APB is most probably acting at a different locus on the synaptic glutamatergic receptors of the horizontal cells or at presynaptic receptors located on the cones themselves.**

Electrophysiological studies of the vertebrate retina have revealed a distinctive excitatory amino acid receptor that is found only on one class of second-order neurons, the depolarizing bipolar cells (DBCs). This receptor is selectively activated by the glutamate analog 2-amino-4-phosphonobutyrate (APB; refs. 1–3). APB mimics the action of the photoreceptor transmitter by hyperpolarizing the DBC membrane, an unusual action for a glutamate analog. Moreover, it has no reported effect on the other second-order neurons (horizontal and hyperpolarizing bipolar cells), where the photoreceptor transmitter and glutamate analogs such as kainate produce a more conventional depolarization (1, 2, 4, 5).

Because of its properties as a selective agonist, APB has been used to pharmacologically dissect functional pathways in the visual system. Beginning at the photoreceptor–bipolar synapse, visual information in the retina is separated into two parallel circuits, the "ON" and "OFF" pathways, which remain segregated throughout the visual system (6, 7). The reported ability of APB to block light responses in DBCs has led to the wide use of APB as a tool for selectively blocking the ON pathway while studying higher-order visual centers (8–10). These studies depend critically on the assumption that APB acts only on DBCs.

Using intracellular recording techniques in the isolated goldfish retina, we now report an additional action of APB that affects the membrane potential and light responses of horizontal cells. APB acted preferentially on cone-driven horizontal cells (CHCs), antagonizing their responses to light, but had no effect on rod-driven horizontal cells (RHCs). Therefore, these results demonstrate a distinct difference in the pharmacology of the rod and cone pathways in the goldfish retina and suggest that the actions of APB in the vertebrate retina might be more complex than was previously thought.

MATERIALS AND METHODS

Preparations. Goldfish [*Carassius auratus*; 4–6 in (10–15 cm)] were obtained from Grassyforks Fisheries (Martinsville, IN), housed in an outdoor pond, and fed twice weekly. For the "dark-adapted" preparations, fish were kept in complete darkness for at least 1.5 hr prior to sacrifice by decapitation. The eyes were enucleated and hemisected, and the retina was then isolated from the pigment epithelium and placed in a 20% solution of hyaluronidase (Wydase, Wyeth) for 20–30 min at 4°C to degrade the vitreous humor. The entire retina was then mounted on an annular-shaped piece of no. 2 filter paper with the receptor side up and placed in the superfusion chamber. Stimulating light was focused onto the retina from below through the hole in the filter paper. The microelectrode was lowered to a position just above the retina and centered in the spot of light. To ensure that the retina remained completely dark-adapted the entire procedure, including the dissection, was performed under infrared illumination using an image converter (Varoscope). For the "light-adapted" preparations, goldfish were placed in complete darkness for no longer than 10 min prior to sacrifice. The dissection was performed with a standard dissecting microscope illuminator, the beam of which was covered by three layers of red acetate paper. The light intensity illuminating the retina was ≈150 $\mu W/cm^2$, as measured with a Minolta LS-100 luminance meter. Retinas were exposed to this illumination for ≈10 min, the duration of a typical isolation procedure. Following the 20-min treatment with hyaluronidase, done in complete darkness, the isolated retina was transferred to the filter paper under red light. Other than the amount of light used, there were no differences between the light and dark dissection procedures.

Superfusion and Recording. The recording chamber was continuously superfused with oxygenated L-15 culture medium (GIBCO) modified to contain the following concentrations of ions: 120 mM NaCl, 1.2 mM $MgSO_4$, 2.5 mM KCl, 2.2 mM $CaCl_2$, 10.0 mM glucose, and was buffered to a pH of 7.8 with 3 mM Hepes. All amino acid analogs as well as Co^{2+} were added without substitution to this solution. Gravity-fed control and test solutions were alternately connected to the recording chamber through a series of valves (Hamilton). The volume of the chamber was ≈0.3 ml. We typically obtained full physiological responses to different test solutions within 45 sec after switching the valves.

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Abbreviations: APB, 2-amino-4-phosphonobutyrate; CHC, cone-driven horizontal cell; RHC, rod-driven horizontal cell.

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Microelectrodes formed from standard omega-dot tubing were pulled on a Brown-Flaming puller to a resistance of 300–500 MΩ and filled with 2 M potassium acetate. Light was projected onto the retina from an optical bench mounted beside the light-tight Faraday recording cage. Neutral density and interference filters (halfwidth, 10 nm) were used to attenuate the light and adjust its color.

RESULTS

The ability to record light responses from different classes of horizontal cell depended on the levels of ambient light that were present before and during isolation of the retina. An example of the light responses of a typical horizontal cell that was obtained with the dark-adapted protocol (see *Materials and Methods*) is shown in Fig. 1*A*. A 10-msec flash of green light, delivered at 0 sec, elicited responses with a time course that was similar to the responses of RHCs in the turtle (12) and dogfish (13). The sensitivity to dim flashes of light was relatively high, an average of 29.5 mV/photon·μm^{-2} ($n = 14$), and similar to the value of 22.8 mV/photon·μm^{-2} obtained in horizontal cells of the dogfish retina (13). We found that the sensitivity of RHCs was lower than the sensitivity of rod-driven DBCs, as has also been reported in dogfish (13). Spectral sensitivity measurements, plotted in Fig. 1*B*, revealed a peak at a wavenumber of 1.90 μm^{-1} (wavelength, 525 nm) close to the absorption maximum for goldfish rhodopsin. The waveform, spectral sensitivity, and absolute sensitivity of the light response suggest that this cell type corresponds physiologically to the intermediate horizontal cell that has been described in the goldfish retina (14, 15) and is thought to receive input exclusively from rods.

In the retina prepared with the light-adapted protocol we primarily obtained two types of horizontal cells, one maximally sensitive to red light, and the other sensitive to green light. A series of responses to different intensities of a 10-msec flash of red light is shown for a red-sensitive cell in Fig. 1*C*. The time course of these responses was much faster than the time course of the RHC responses, with a time peak of ≈100 msec, similar to the responses of CHCs in the turtle retina (16). The flash sensitivity of these cells was much lower than for the RHCs, ≈150 μV/photon·μm^{-2} ($n = 11$), somewhat below the value of 345 μV/photon·μm^{-2} obtained in the turtle (16). Spectral sensitivity measurements of the responses peaked at either 608 nm (cell in Fig. 1*D*) or 530 nm (green-sensitive cells; data not shown), close to the absorption maxima of the red and green cones, respectively.

In the case of red-sensitive horizontal cells, spectral sensitivity curves provide evidence that the rod inputs contributed at most only a very small component to the light response obtained in the light-prepared retinas. Spectral sensitivity alone is not sufficient to distinguish between rod and green cone input, as they are maximally sensitive at nearly the same wavelength. However, the flash sensitivity (mV per incident photon) for green-sensitive cells obtained by the light-adaptation protocol was 2–3 log units lower than the RHCs. In addition, the time course of the responses was much more rapid than that of the RHCs (compare Figs. 1 and 3), which would be expected on the basis of the much faster responses observed for green cones (17). We therefore believe that the light responses we encountered in retinas prepared by the light-adapted protocol originated from cones and that the cells we recorded from probably correspond to Stell's H1 and H2 cells, which are contacted by red- and green-sensitive cones but not at all by rods (18).

Previous studies in the mudpuppy (2), dogfish (1), and rabbit (4, 6) retinae have shown that APB acts specifically on depolarizing bipolar cells and has no effect on horizontal cells. To test whether this same specificity prevailed in the goldfish retina, we measured the effects of APB on RHCs and CHCs.

Fig. 2 shows that in the dark-adapted retina, APB had little or no effect on the membrane potential or light response of RHCs. At the time indicated by the first arrow, the control solution was switched to a solution containing 10 μM APB. There was little change in membrane potential. Fig. 2*B* demonstrates that there was also no substantial difference between the averaged light response in control solution and the response that was obtained during superfusion of APB. Under the same dark-adapted conditions, the rod-driven DBCs were hyperpolarized by ≈10 mV by 2 μM APB (19). These results are consistent with previous studies, which suggested that postsynaptic receptors on horizontal cells and DBCs are pharmacologically distinct.

More unexpected were the actions of APB on CHCs, as illustrated in Fig. 3*A*. At the time indicated by the first arrow, 10 μM APB was applied to the retina. The cell membrane hyperpolarized by 25 mV to a potential of about −60 mV. Following return to the control solution, the membrane potential returned to its original value. During superfusion of APB, the light response amplitude was attenuated to less than one-third of its original size (Fig. 3*B*). The cell shown here was driven by green-sensitive cones, but APB had similar effects on all classes of CHCs that we examined. However,

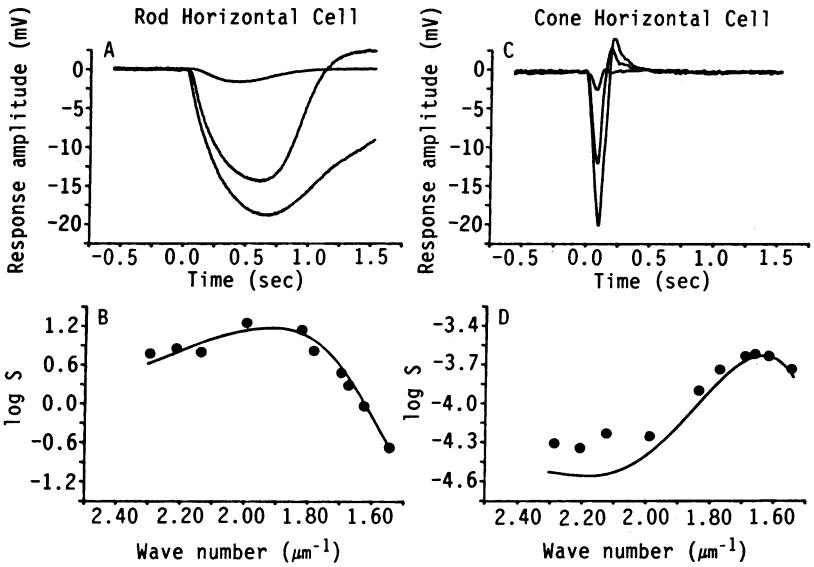

FIG. 1. Properties of horizontal cells in light- and dark-prepared retinas. (*A*) Responses of a horizontal cell in a dark-prepared retina to 10-msec flashes of 550-nm light at intensities of 0.09, 0.90, and 8.97 photons/μm^2 per flash. The stimulus was presented at 0 sec. (*B*) Data points are the average spectral sensitivity for seven horizontal cells. The inverse of the logarithm of the number of photons required to elicit a 3-mV light response is plotted on the *y* axis, while the wavenumber (inverse of wavelength) is plotted on the *x* axis. The smooth curve is the nomogram for an A_2-based pigment with a peak sensitivity at 523 nm and was generated with a polynomial that provided a good fit to the A_2 nomogram (11). (*C*) Responses of a horizontal cell recorded in a light-adapted retina to 10-msec flashes of 600-nm light at intensities of 62, 3140, and 3.1 × 10^4 photons/μm^2 per flash. (*D*) The average sensitivity of six horizontal cells as a function of wavelength. The nomogram is a polynomial for an A_2 retinol with a peak sensitivity at 618 nm (11).

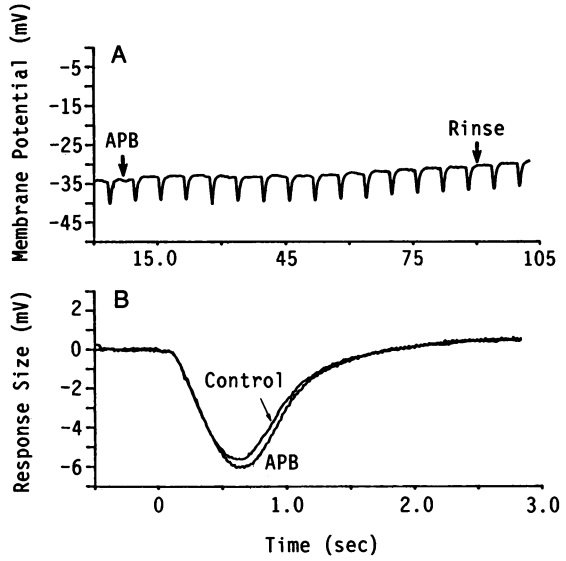

FIG. 2. APB has no effect on RHCs. (*A*) Intracellular recording of a RHC. The downward deflections are responses to a 10-msec flash of 550-nm light containing 3.7 photons/μm^2, delivered every 6 sec. The control solution was switched to one containing 10 μM APB at the time indicated by the first arrow, and then back again at the second arrow. (*B*) The average light response during superfusion of control (five responses) and APB (six responses) solution is shown on a magnified gain and expanded time base. APB had no substantial effect on either light response or membrane potential.

it should be noted that in some cells (30%), we observed no attenuation of the light response and no hyperpolarization in the presence of APB. The reason for such a variable action of APB is unclear, but it was not correlated with any specific class of CHC.

Evidence in favor of an action of APB on the cone–CHC synapse and against an action at a nonsynaptic receptor came from comparing the effects of Co^{2+} and APB on the light response and membrane potential of the same CHC. Fig. 4*A*

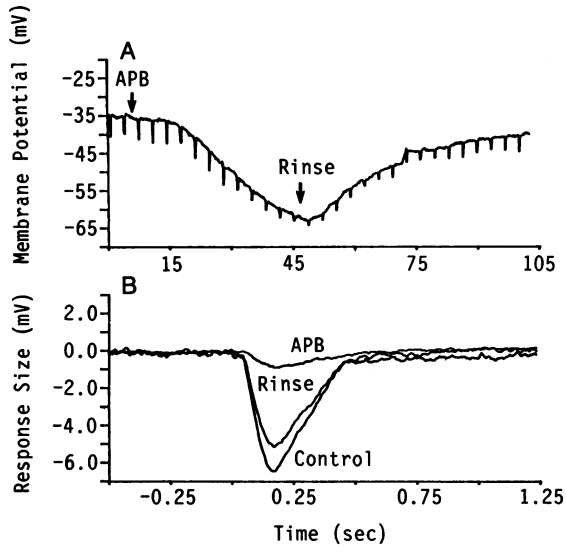

FIG. 3. Responses of CHCs are blocked by APB. (*A*) The same experiment as in Fig. 2 except the recording was from a green-sensitive CHC obtained from a light-adapted retina. The cell responded to application of 10 μM APB with a hyperpolarization and a reduction in the light response. The effects were reversed upon rinse out of APB. (*B*) Light responses before, during, and after application of APB as indicated. The stimulus was a 10-msec flash of 650-nm light delivering $\approx 1.7 \times 10^4$ photons/μm^2.

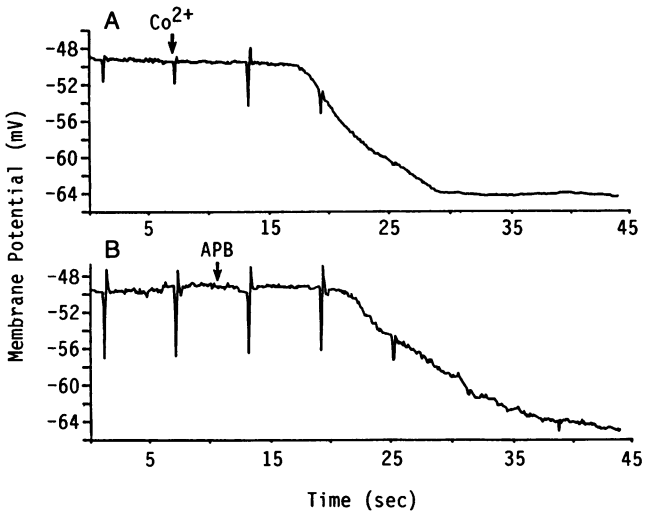

FIG. 4. Cobalt and APB have similar effects. (*A*) Recording from a red-sensitive CHC from a light-adapted retina. At the arrow, 1 mM Co^{2+}, dissolved in the control solution, was applied. The cell hyperpolarized and the light response was completely blocked. Membrane potential and light response recovered following rinse out (data not shown). The increase in the size of light response following Co^{2+} application was produced by an increase in the stimulus duration from 10 to 50 msec. At 50 msec, the flash contained $\approx 3.8 \times 10^4$ photons/μm^2. (*B*) Response of the same cell to application of 10 μM APB (arrow). Following rinse out, the cell recovered once again. Stimulus was a 50-msec flash of 650-nm light containing $\approx 1.5 \times 10^4$ photons/μm^2.

demonstrates that 1 mM Co^{2+}, added to the normal superfusion solution, hyperpolarized the cell and eliminated the light response. At this concentration, cobalt has been shown to block the release of photoreceptor transmitter to the same degree as saturating intensities of light (20). Following recovery from Co^{2+}, the control solution was switched to one containing 10 μM APB (Fig. 4*B*). The cell was hyperpolarized by Co^{2+} or APB to nearly the same potential, which is presumed to be the membrane potential of the cell in the absence of significant synaptic input from photoreceptors. The effects of Co^{2+} and APB were not additive. Application of Co^{2+} alone produced the same changes in membrane potential and light response as the application of Co^{2+} and APB together. These experiments suggest that APB is as effective a synaptic blocker as cobalt at the cone–CHC synapse.

To examine the mechanism and site of APB antagonism, we tested the ability of APB to block the postsynaptic action of two photoreceptor transmitter agonists, glutamate and kainate. Kainate has been shown to be a potent agonist at the CHC postsynaptic receptor, where it depolarizes the cell membrane and blocks the light response (4, 5, 21, 22). Fig. 5*A* illustrates the effect of 7 μM kainate on the membrane potential of a red-sensitive CHC. Following application of kainate, the cell depolarized to about 0 mV. The transient depolarization at the beginning of the response is probably due to the activation of a Ca^{2+} current (23). Fig. 5*B* demonstrates the effect of 7 μM kainate, together with 10 μM APB, on the same cell. Kainate produced a response with about the same size and time course in the presence and absence of APB, indicating that APB did not significantly antagonize the CHC responses to kainate. Similar results were obtained when the concentration of APB was raised to 100 μM, a concentration that is 50 times greater than necessary to hyperpolarize the CHC and block the light response.

Responses to glutamate (2 mM) in the absence and presence of APB were also examined. The depolarizing action of glutamate was not blocked or significantly attenuated by

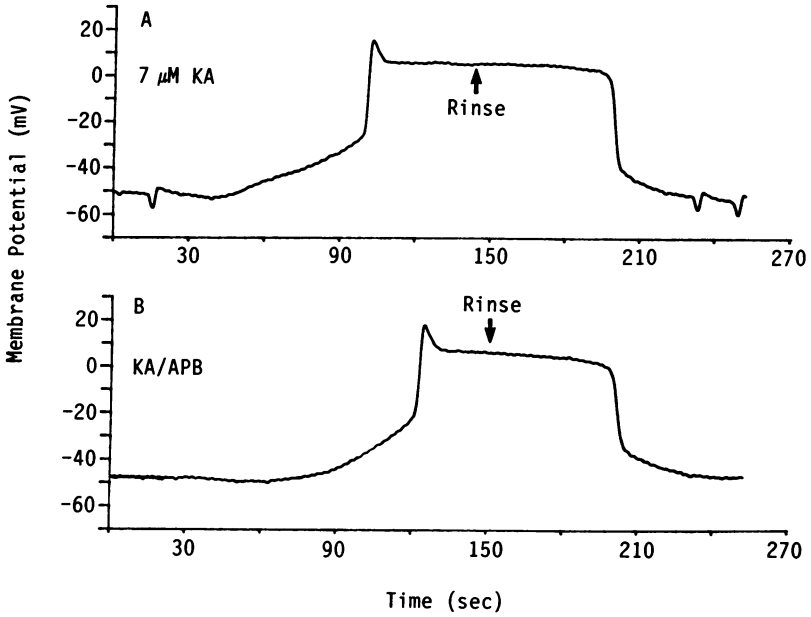

FIG. 5. APB does not block the effect of kainate (KA). (*A*) Response of a red-sensitive horizontal cell. At 15 sec, the superfusion solution was switched to 7 μM kainate. The cell depolarized slowly and then more rapidly. During this time, the light response was nearly blocked (data not shown). At the arrow, the superfusion was switched back to control. The figure was constructed by using a low-gain recording that was low-pass filtered at 2.5 Hz to remove tape recorder noise. The three downward deflections in *A* are filtered responses to a light stimulus of 1-sec duration and were blocked by kainate. (*B*) Same experiment as in *A*, with 10 μM APB added with the kainate. Note that the cell is driven to nearly the same potential with nearly the same time course in the presence and absence of APB, suggesting that APB has little or no effect on the action of kainate.

APB. The failure of APB to block the action of either glutamate or kainate suggests that APB acts at a different site than glutamate or kainate.

DISCUSSION

The main finding of this study is that APB antagonizes synaptic transmission from cones to CHCs but does not interfere with transmission from rods to RHCs. We have previously demonstrated that in goldfish retina APB acts as a rod transmitter agonist on DBCs and that it effectively blocks transmission from rods to rod-driven DBCs (19). The present study, taken together with our previous results, indicates that there are at least two distinct sites of APB action in this retina. In one case, the action is directly on the DBCs; in the other case, it is on either the cones or the CHCs.

Our results differ from studies in other species in which APB was found to be ineffective on all types of horizontal cells (1, 2, 4, 5). The discrepancies might be due to species differences or to some other factor, such as the adaptation state of the retina. In some (about one-third) of the CHCs that we tested, the amplitude of the light response was not reduced significantly by APB. These APB-insensitive cells were found in retinas that were exceptionally light adapted. It is not clear how the adaptation state of the retina might affect the responses to APB or whether it can account for the discrepancies between this and previous studies.

APB has been shown to depress synaptic transmission but not block the action of exogenous glutamate and various glutamate analogs in other areas of the brain, such as the pyriform cortex (24, 25), the lateral perforant pathway (26), and the spinal cord (27). While there was no clear evidence for a precise mechanism or site of action, the results in these previous studies as well as the present one are consistent with a presynaptic effect of APB. Recent electrophysiological evidence has shown that there is an excitatory amino acid receptor-mediated conductance in tiger salamander cones (28). According to estimates from previous studies of synaptic transmission from photoreceptors to second-order cells, a 2- to 4-mV hyperpolarization of the photoreceptor can produce an *e*-fold reduction in transmitter release (29, 30). APB-induced suppression of transmitter release from cones would therefore only require a small hyperpolarization of the cone membrane, an action of APB that has already been demonstrated in rod-driven DBCs (19). However, in spite of the above considerations, the possibility that APB binds to a different postsynaptic site than either glutamate or kainate cannot be ruled out (25).

Since it was first postulated that APB selectively blocks the ON pathway at the DBC, a number of investigators have used APB to study the functional circuitry of synaptic inputs to higher-order pathways of the visual system in mammals, including monkey (7, 9), cat (10, 31), and rabbit (8). In these studies, the responses of neurons in the lateral geniculate nucleus or cortex were recorded during intraocular injection of APB. Similarly, blockade of the b-wave component of the electroretinogram by APB has led to speculation that the b-wave can be used to monitor ON bipolar activity (32). However, the interpretation of such experiments depends on the assumption that APB acts only on the ON pathway. Our results suggest that under certain conditions, APB can act on both the ON and OFF pathways. Two recent studies, one on goldfish (33) and the other on cat (34), support this hypothesis by showing that APB can modulate the responsiveness of both ON and OFF ganglion cells to light. Making detailed models of neuronal circuity based on the pharmacological actions of APB might therefore be premature, as the actions of this compound appear to be less specific than was previously thought.

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