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Co-release of Acetylcholine and GABA by the Starburst Amacrine Cells

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Rabbit retinas were isolated from the eye and maintained *in vitro.* When they were incubated for 60 min in the presence of ^{3}H-GABA, subsequent autoradiography showed radioactivity to be present primarily in amacrine cells. Under these conditions, most of the radioactivity contained in the retinas remained in the chemical form of GABA. Autoradiography and immunohistochemistry of alternate sections showed the amacrine cells that accumulate ^{3}H-GABA to be the same cells that contain endogenous GABA immunoreactivity. These include the starburst cells, the indoleamine-accumulating cells, and other, as yet unidentified amacrine cells. The localization confirms previous immunohistochemical findings. When retinas containing ^{3}H-GABA were exposed to elevated concentrations of K^+, their content of ^{3}H-GABA decreased. Autoradiography showed a reduced ^{3}H-GABA content in all of the cells that contained ^{3}H-GABA. Since those include the starburst cells, previously shown to be cholinergic, the finding demonstrates that the starburst cells release both ACh and GABA.

Retinas simultaneously labeled with ^{14}C-GABA and ^{3}H-ACh were superfused, and the release of radioactive compounds from the retina was studied. Depolarization by elevated K^+ caused an increased recovery of both ACh and GABA in the superfusate, but the predominant mechanisms of their release appeared to be different. The stimulated release of ACh was entirely Ca^{2+} dependent, while the release of radioactivity originating from GABA was much less so. A concentration-dependent counterflux (homoexchange) of intracellular GABA was demonstrated by raising the extracellular concentration of GABA (or nipecotic acid). These results suggest that a large outward flux of GABA occurs via the GABA transporter, probably by the potential-sensitive mechanism studied by Schwartz (1982, 1987).

Stimulation of double-labeled retinas by flashing light or moving bars always increased the release of ACh, and the release was entirely dependent on the presence of extracellular Ca^{2+}. Stimulation with light never caused a detectable release of GABA. This was unexpected, since the two neurotransmitters are present in the same amacrine cells: stimulation adequate to release one neurotransmitter should release both. Control experiments showed the following: (1) GABA synthesized endogenously from radiolabeled glutamate was released in the same overall way as GABA accumulated from the medium; (2) inhibition of GABA-transaminase suppressed the degradation of GABA within the retina but did not unmask a light-stimulated GABA release; (3) saturating concentrations of agents that affect GABA reuptake (nipecotic acid, SKF 89976A, unlabeled GABA) increased the recovery of radioactive GABA in the perfusate but did not unmask a light-stimulated release of GABA; and (4) treatment with APB did not reveal a light-evoked response, indicating that its absence was not due to counterbalancing of the retina's ON and OFF responses.

An interpretation of these results is that the carrier-mediated release of GABA is greater, overall, than GABA's secretion in synaptic vesicles, so that the carrier-mediated component overwhelms vesicular release when the whole tissue is studied. If this is so, however, the carrier-mediated release must be relatively insensitive to the membrane potential, so that release is detectably increased by the large, long-lasting depolarizations induced by rises in extracellular K^+, and not to the small (and brief) depolarizations caused by stimulation of the retina with light.

The starburst amacrine cells, originally identified by their synthesis and release of ACh, have been shown by three independent laboratories to contain GABA as well as ACh. They form part of a larger family of GABA-containing retinal neurons that includes several other amacrine cells (Masland and Mills, 1979; Vaney et al., 1981; Famiglietti, 1983; Tauchi and Masland, 1984; Brecha et al., 1988; Kosaka et al., 1988; Vaney and Young, 1988). The colocalization of GABA and ACh in the starburst cells raises questions fundamental to the physiological role of those cells in the retina. For example, Vaney (1990) has speculated that the coordinated releases of both an excitatory and an inhibitory transmitter could allow the starburst cells to create directional selectivity in certain retinal ganglion cells.

Some simpler questions, however, remain univestigated. One of them is whether or not the two neurotransmitters are secreted in parallel. Are ACh and GABA always released in concert, or can their rates of secretion diverge? What physiological events drive their releases? In the work reported here, we measured the release of ACh and GABA from retinas maintained *in vitro.* Because the rabbit retina contains a single type of ACh-synthesizing cell, the release of ACh from the whole tissue must reflect the activity of those cells, and light-stimulated release of ACh is readily measured (Masland and Livingstone, 1976; Massey

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and Neal, 1979; Neal and Massey, 1980; Massey and Redburn, 1982; Masland et al., 1984b; Cunningham and Neal, 1985; Masland and Cassidy, 1987). Except for one unsubstantiated report (Bauer, 1978), a light-stimulated release of GABA has not been demonstrable, but its cellular localization and release by K^+ depolarization have been widely investigated (for review, see Redburn et al., 1983).

To study the co-release of ACh and GABA, we labeled them with different isotopes, so that they could be simultaneously detected in media that had superfused the retina. The double-labeling procedure served as an internal control, permitting sensitive comparisons between the behaviors of the two neurotransmitters. We found that the overall releases of ACh and GABA behaved differently. Although the two neurotransmitters are colocalized in a large group of amacrine cells, a release of ACh was readily evoked by stimulation with light, while a release of GABA was not.

A short report of part of this work has been published (O'Malley and Masland, 1989). Here we report further attempts to evoke a co-release of ACh and GABA, a new series of control experiments, a demonstration of GABA's counterflux in response to extracellular substrates, and an analysis of GABA's chemical fate and movement between cells under our experimental conditions. This last seemed particularly important. Although the double-labeling procedure had crucial advantages for these studies, the use of a radiochemical method of detecting GABA required special care that the intended cells contained the labeled neurotransmitter.

Materials and Methods

Labeling and superfusion. Forty-seven retinas were studied, most of them for experiments that lasted several hours and included a variety of experimental manipulations.

Procedures for isolating retinas from New Zealand White rabbits and maintaining them *in vitro* were based on those previously described (Ames and Nesbett, 1981; Masland et al., 1984a,b). All were carried out under dim red illumination. Rabbits were dark adapted for at least 30 min, anesthetized with intravenous pentobarbital (30 mg/kg), and brought to surgical anesthesia with ether. The eye was enucleated and hemisected, and the retina was teased away from the sclera while immersed in oxygenated Ames medium (Sigma). The electrolytes and organic components in this medium (amino acids, vitamins, and cofactors) were present at concentrations designed to approximate mammalian CSF. Stringent precautions were taken to prevent possibly toxic substances from coming into contact with the tissue.

Once dissected, retinas were placed in rocking tubes ("boats") that contained 6 ml of control medium equilibrated with 95% O_2, 5% CO_2 (Ames and Nesbett, 1981). Retinas were preincubated in control medium for 10 min prior to radiolabeling to allow for recovery from the isolation procedures. They were labeled by incubation for 1 hr at 37°C in medium containing the following concentrations of applicable radiolabels: methyl-^{3}H-choline, 0.1 μM (80 Ci/mmol); ^{14}C-U-GABA, 9.0 μM (238 mCi/mmol); ^{3}H-[2,3]-GABA, 0.11 μM (92 Ci/mmol); and L-[^{14}C(U)]-glutamate, 5.8 μM (292 mCi/mmol) (all from New England Nuclear). Low-calcium medium, which contained an elevated concentration of magnesium (20 mM) and a reduced concentration of calcium (0.2 mM), was made by isotonic substitution for NaCl. Other media were made by adding drugs or KCl directly to control or low-calcium medium as indicated. If the metabolism of GABA was to be inhibited, retinas were preincubated for 30 min with 200 μM γ-vinyl GABA (GVG) (gift of Merrell Dow), which was also present during labeling. SKF 89976-A was donated by Smith Kline French Laboratories.

For superfusion experiments, a patch of retina was mounted in a Lucite and glass chamber. The base of the chamber consisted of a coherent fiber optic bundle ½ inch in diameter (Parker Products, Boston, MA) ground to the approximate curvature of the retina. The walls and top of the chamber were of polished acrylic; they sealed at an O-ring around the fiber optic bundle, creating an enclosed volume of 0.5 ml. In overall geometry, the chamber was similar to one shown by Masland et al. (1984b). The patch of retina was roughly 5 × 5 mm and was taken from a region centered about 5 mm below the visual streak. Diffuse light or focused images were delivered to one end of the fiber optic bundle via an optical bench. The intensity and timing of stimulation were controlled by neutral density wedges and electromagnetic shutters. The individual fibers had a diameter of 6 μm, ensuring resolution adequate for these present experiments.

Retinas were mounted in the superfusion chamber, and after a 20–30 min recovery period, fractions were collected at 30 sec intervals (flow rate, 4 ml/min; 2 ml fractions). A 1 ml aliquot of each fraction was counted, and ^{3}H and ^{14}C were determined by conventional methods. Counting efficiencies were 28% for ^{3}H and 66% for ^{14}C. The release of ^{3}H-acetylcholine into the superfusing medium was monitored by counting the total radioactivity released into the superfusate. In experiments carried out without use of an anticholinesterase, as these were, most of that radioactivity is in the form of choline, the released acetylcholine having been hydrolyzed within the tissue. In previous experiments, we systematically analyzed the radioactive choline metabolites released, with physostigmine or neostigmine present. Somewhat more than half of the total radioactivity released at rest (in the dark) was acetylcholine, with the remainder as choline. The increase in release following stimulation by light was virtually all due to increased release of radioactive acetylcholine (see Masland and Cassidy, 1987, their Fig. 5). These results agree well with those of others who have made a similar comparison (Neal and Massey, 1980; Massey and Redburn, 1982). Although in some ways it might be preferable to measure acetylcholine directly, the indirect method allowed more frequent sampling of the superfusate and longer runs of experiments. More importantly, it allowed the experiments to be done without the use of anticholinesterases, which create an artificially elevated level of acetylcholine within the tissue and unnatural electrical activity in its neurons (Masland and Wigton, 1942; Masland and Ames, 1976; Ariel and Daw, 1982; Masland and Cassidy, 1987).

Selected fractions were analyzed biochemically in some experiments. In these cases, each period of photic stimulation lasted 5 min (10 samples). Groups of 10 fractions immediately before, during, and after the stimulation were pooled and their radiochemical contents analyzed. Separation of radiolabeled compounds was done by high-voltage paper electrophoresis (Potter and Murphy, 1967; Hildebrand et al., 1971; Masland and Livingstone, 1976), with authentic standards of radiolabeled ACh, GABA, choline, and/or glutamate run in adjacent lanes.

Fractions from the superfusion experiments were too dilute to be electrophoresed directly and were thus concentrated on ion exchange columns (see Iversen and Kravitz, 1968). Groups of 10 fractions (1 ml each) were combined and loaded onto 2.2 ml Dowex 50W cation exchange columns that had been equilibrated with 0.5 *N* HCl. After washing with 3 ml distilled water, radioactive GABA was eluted with 1 M ammonium hydroxide; the eluant was collected in 0.7 ml fractions, and peak fractions were evaporated under a stream of nitrogen, resuspended in formate/acetate buffer, and analyzed by high-voltage paper electrophoresis. When authentic ^{14}C-GABA standards were loaded onto the columns, more than 97% was recovered, even in the presence of 1 mM unlabeled GABA. The ^{14}C-GABA standard recovered from the columns ran as a single peak when electrophoresed. We calculated the percentage of radioactivity in the superfusate that was GABA by multiplying the percentage of the total radioactivity in the sample that bound to the Dowex column times the fraction of the bound radioactivity that migrated with authentic GABA upon electrophoresis. In experiments where metabolism of GABA proceeded unchecked, less than 2% of the radioactivity ran as GABA, confirming that the radioactivity that normally migrated with GABA was GABA.

All of the superfusion experiments presented here used ^{14}C-labeled GABA. Because ^{3}H-GABA was used for autoradiography, analyses of ^{3}H-labeled compounds in the tissue and medium are shown in Figures 9, *B* and *C*, and 10, *A* and *B*. However, metabolites of ^{3}H-GABA include ^3H_2O (Gardner and Richards, 1981), and ^{14}C-GABA was sometimes used as a check of the major conclusions (see Figs. 9*A*; 10*C,D*). (Although losses to ^3H_2O probably occurred, ^{3}H-metabolites were readily detected; see Fig. 10*A,B*.) The identity of the metabolites was not studied further.

Autoradiography. Retinas labeled with ^{3}H-GABA were rinsed for 5 min in 15 ml of room temperature Ames medium to clear radiolabeled GABA from the extracellular space. The retinas were fixed by immersion in 3% glutaraldehyde and processed for autoradiography by standard methods. For double labeling with ^{3}H-GABA and 4,6-diamidino-2-phenylindole (DAPI), intraocular injection of DAPI was performed as described (Masland et al., 1984a). Since glutaraldehyde quenches the

fluorescence of DAPI, 4% paraformaldehyde was used for double-label experiments involving DAPI. The autoradiographic labeling for GABA was less sharp with paraformaldehyde fixation than with glutaraldehyde, but the results were otherwise identical.

Immunohistochemistry and analysis of serial sections. When autoradiography was combined with immunohistochemistry for endogenous GABA, retinas were incubated in ^{3}H-GABA for 1 hr and rinsed as described above. Following the rinse, relieving cuts were made around the perimeter of the retina and it was flattened between sheets of moist filter paper (Tauchi and Masland, 1984; Sandell and Masland, 1986). The filter paper sandwich containing the retina was immersed in 5% glutaraldehyde in 0.1 M PO_4 buffer (pH 7.4) at 4°C overnight. The following day, the sandwich was rinsed and dehydrated in ascending alcohols; the retina was freed from the filter paper, immersed in acetone for 2 × 10 min, and infiltrated with MedCast epoxy resin (Ted Pella, Inc.). Pieces of retina were cut from the whole-mount and embedded in MedCast for vertical sections. The resin was polymerized overnight at 60°C. Serial 1 μM sections were processed for autoradiography or deplasticized and immunoreacted using rabbit anti-GABA (Chemicon) and colloidal gold intensified with silver (Moremans et al., 1984; Marc et al., 1990).

When ^{3}H-GABA autoradiography was combined with serotonin immunohistochemistry, the labeling boats contained 1 μM serotonin (Sigma #H7752) in addition to ^{3}H-GABA. The retinas were incubated for 1 hr, rinsed, and fixed on ice for 1 hr in 5% glutaraldehyde in 0.1 M PO_4 buffer (pH 7.4). Immunohistochemistry for serotonin was carried out on whole-mounts, using standard methods with rabbit anti-serotonin (Pelfreeze #P40601-B) as primary antibody, followed by an avidin-peroxidase-peroxidase procedure (Vectastain ABC, Vector Labs). Diaminobenzidine was used as the chromagen. Stained whole-mounts were embedded in JB4, sectioned at 5 μM and processed for autoradiography.

Labeling cells with Lucifer yellow. The shape of the Müller cells (see Fig. 9) was visualized by filling with Lucifer yellow CH (Sarthy et al., 1982). Retinas were isolated as usual and placed in Ca/Mg-free Ames medium containing 5 mM EGTA (Sigma). After rocking for 15 min at 36°C, Lucifer yellow CH (Aldrich) was added to a final concentration of 0.4%. The retinas were incubated in the Lucifer yellow solution for 20 min and then rinsed for 3 × 5 min in Ca/Mg-free medium with EGTA. The retinas were fixed overnight in 4% paraformaldehyde in PO_4 buffer. Pieces of retinas were embedded in JB4, and sections (4–10 μm) were cut on a rotary microtome. We do not know why some cells were selectively labeled by this procedure. Quite possibly the unphysiological conditions of the incubation make membranes of some cells leaky, allowing the dye to enter.

Autoradiographic observation of ^{3}H-GABA release. Retinas were labeled with ^{3}H-GABA for 1 hr. After a brief rinse, the retinas were cut in half along the dorsal-ventral axis. The half-retinas were transferred through a series of three 6 ml boats containing either control medium, for one half-retina, or releasing medium (20 mM KCl, 1 mM GABA), for the other half-retina. They were then rinsed, fixed in 3% glutaraldehyde, and processed for autoradiography. The labeling boat was counted before and after incubation of the retina to determine the amount of ^{3}H-GABA accumulated by the retina. The initial ^{3}H-GABA content of each half-retina was estimated by assuming each contained half of the total accumulated ^{3}H-GABA; the sizes of the half retinas differed by less than 7%. The amount of radioactivity released during each of the subsequent incubations was determined by counting aliquots of each boat. The fraction released was calculated as the total dpm released into the three boats compared with the total originally accumulated.

Results

Our initial findings are summarized by Figure 1, which shows the release of tritium (originating as ^{3}H-ACh) and ^{14}C (originating as ^{14}C-GABA) from a superfused retina. Both radiolabels were released into the superfusate. The release of ACh was stimulated by flashing light or moving bars, and it increased even more during superfusion with medium containing 30 mM K^+. The release of GABA was also increased by elevated K^+, but it never increased in response to light. The stimulated release of ACh was entirely prevented by incubation in low-Ca^{2+} medium, but the release of GABA was unaffected (see below). Nipecotic acid caused an increased recovery of GABA, as expected for an agent that competes for GABA reuptake, but there was still no light-stimulated release of GABA.

With the experiments described below, we sought an explanation for the differences between the releases of ACh and of GABA.

Cellular location of labeled GABA

The radioactive compounds released in these experiments had been accumulated by the retina during prelabeling. It seemed important to confirm that the expected cells were labeled, and that they in fact included the starburst amacrine cells. Freshly isolated rabbit retinas were incubated with ^{3}H-GABA. At an interval from 15 min to 2 hr, half-retinas were removed from the labeling boat, rinsed briefly to clear the radiolabel from the extracellular space, and fixed in 3% glutaraldehyde. Sections from the half-retinas were processed for autoradiography (Fig. 2). Other samples were extracted into acid and their radiochemical contents determined biochemically.

After 15 min of incubation in the presence of ^{3}H-GABA (Fig. 2*A*), the predominant labeling seen in autoradiographs was a diffuse pattern attributable to uptake of GABA by Müller cells: the density of silver grains paralleled the extent of the Müller cells and was influenced by manipulations that affect a Müller cell enzyme (see below). When the duration of the incubation with ^{3}H-GABA was increased, labeled neurons began to be visible. After a 30 min incubation, labeling over the inner plexiform layer was heavier and labeled cell bodies began to appear at the base of the inner nuclear layer; Müller cell labeling was diminished at this point. After a 60 min incubation, the pattern of labeling was that characteristic of neurons: the inner plexiform layer and small, round cell bodies with the position of amacrine cells account for nearly all of the radiolabeling (Fig. 2*B*). (Displaced amacrine cells and a few ganglion cells also contained ^{3}H-GABA.) This labeling was stable: the pattern of labeling after a 135 min incubation was indistinguishable from the pattern after a 60 min incubation. These results are consistent with those observed when radioactive GABA is injected into the eye *in vivo* (Ehinger and Falck, 1971; Marshall and Voaden, 1975; Ehinger, 1977; Agardh and Ehinger, 1982; Agardh and Bauer, 1984; Redburn and Madtes, 1986; Mosinger and Yazulla, 1987; Yu et al., 1988).

In other experiments we incubated isolated rabbit retinas in a tracer concentration of highly radioactive GABA for 1 hr, then fixed and sectioned them serially at 1.0 μm. Alternate sections were processed for immunohistochemistry using antibodies against GABA-glutaraldehyde conjugates (Fig. 3*A*) or processed for autoradiography (Fig. 3*B*). The number of GABA-containing cells was the same as in retinas that had not been exposed to exogenous (i.e., radiolabeled) GABA, indicating that the radioactive tracer, which was present in very low concentration, had mixed with endogenous GABA normally present. For a few cells only one of the two labels was observed, but this was presumably due to grazing sectioning. Double labeling was very frequent (Fig. 3*A,B*, arrows). We conclude that the cells that accumulate GABA from the extracellular medium are those that normally contain it. Although GABA is accumulated by mouse and rabbit horizontal cells early in development (Schnitzer and Rusoff, 1984; Redburn and Madtes, 1986; Messersmith and Redburn, 1990), there is no significant accumulation in horizontal cells in the adult rabbit (Agardh et al., 1986, 1987; Mosinger and Yazulla, 1985; Redburn and Madtes, 1986; Perez and Bruun,

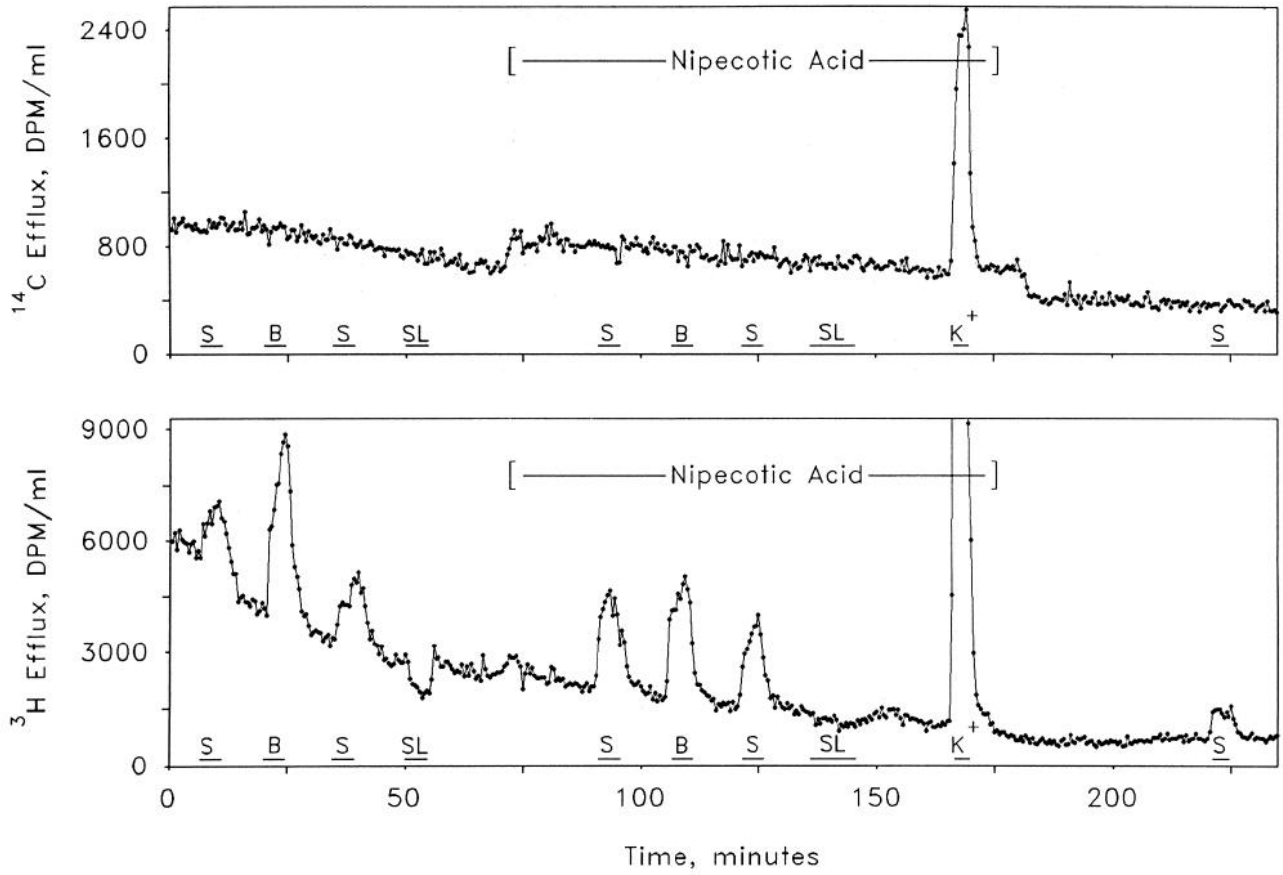

Figure 1. Simultaneously measured releases of ^{3}H from ACh (*bottom*) and ^{14}C from GABA (*top*) from the retina. A retina labeled with ^{3}H-choline and ^{14}C-GABA was stimulated with light and exposed to medium containing 30 mM K^+. Photic stimulation with steady light (*SL*), a 3 Hz flashing strobe (*S*), or moving bars (*B*; pattern of 800-μm-wide black and white bars moving at 1600 μm/sec) or 30 mM KCl (K^+) evoked increases in ACh release. The intensity of the moving bars was 0.8 relative log units; for the steady light it was 0.6 and 2.8 log units. Where indicated, nipecotic acid was present at 100 μM.

1987; see Osborne et al., 1986, for an exception). A low level of endogenous GABA is probably present in rabbit horizontal cells (Wässle and Chun, 1988, 1989; J. H. Sandell and R. H. Masland, unpublished observations). However, it is irrelevant to the present studies since in these experiments we examined the release of radioactive (exogenously administered) GABA, which accumulated only in amacrine cells (Fig. 2*B*).

In the ganglion cell layer of the rabbit retina, the fluorescent dye DAPI selectively labels the "displaced" cholinergic cells (Masland et al., 1984a). It was injected intravitreally and the

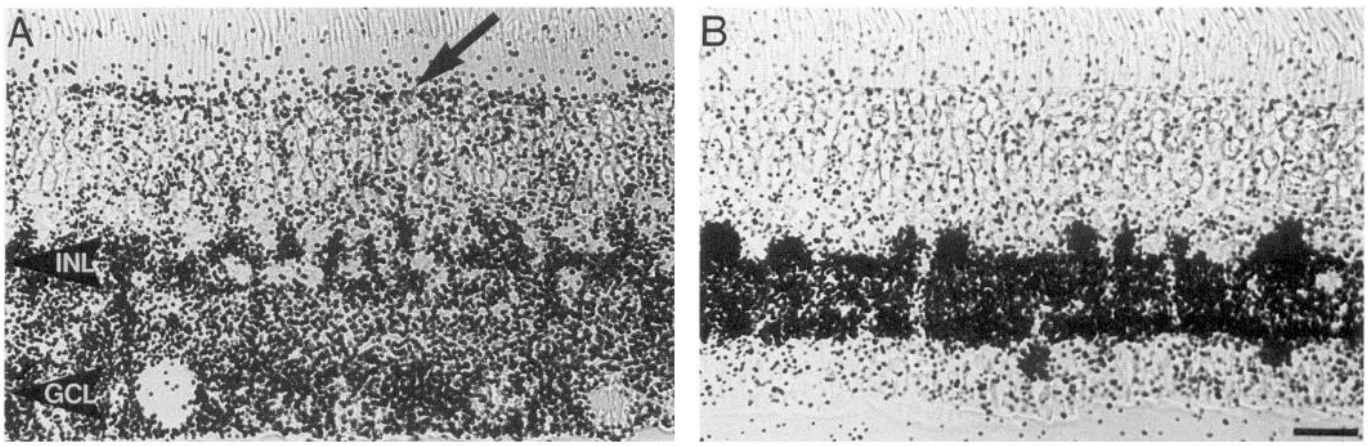

Figure 2. Change in autoradiographic labeling with increasing time of incubation. Retinas were labeled with ^{3}H-GABA. After 15 min (*A*) or 60 min (*B*), half-retinas were removed from the labeling boats, rinsed briefly, fixed in 3% glutaraldehyde, and processed for autoradiography. *Arrows* in *A* show the inner nuclear layer (*INL*), the ganglion cell layer (*GCL*), and the accumulation of label just below the outer limiting membrane. Both sections were exposed for 13 d. *GCL*, granular cell layer; *INL*, inner nuclear layer. Scale bar, 20 μm.

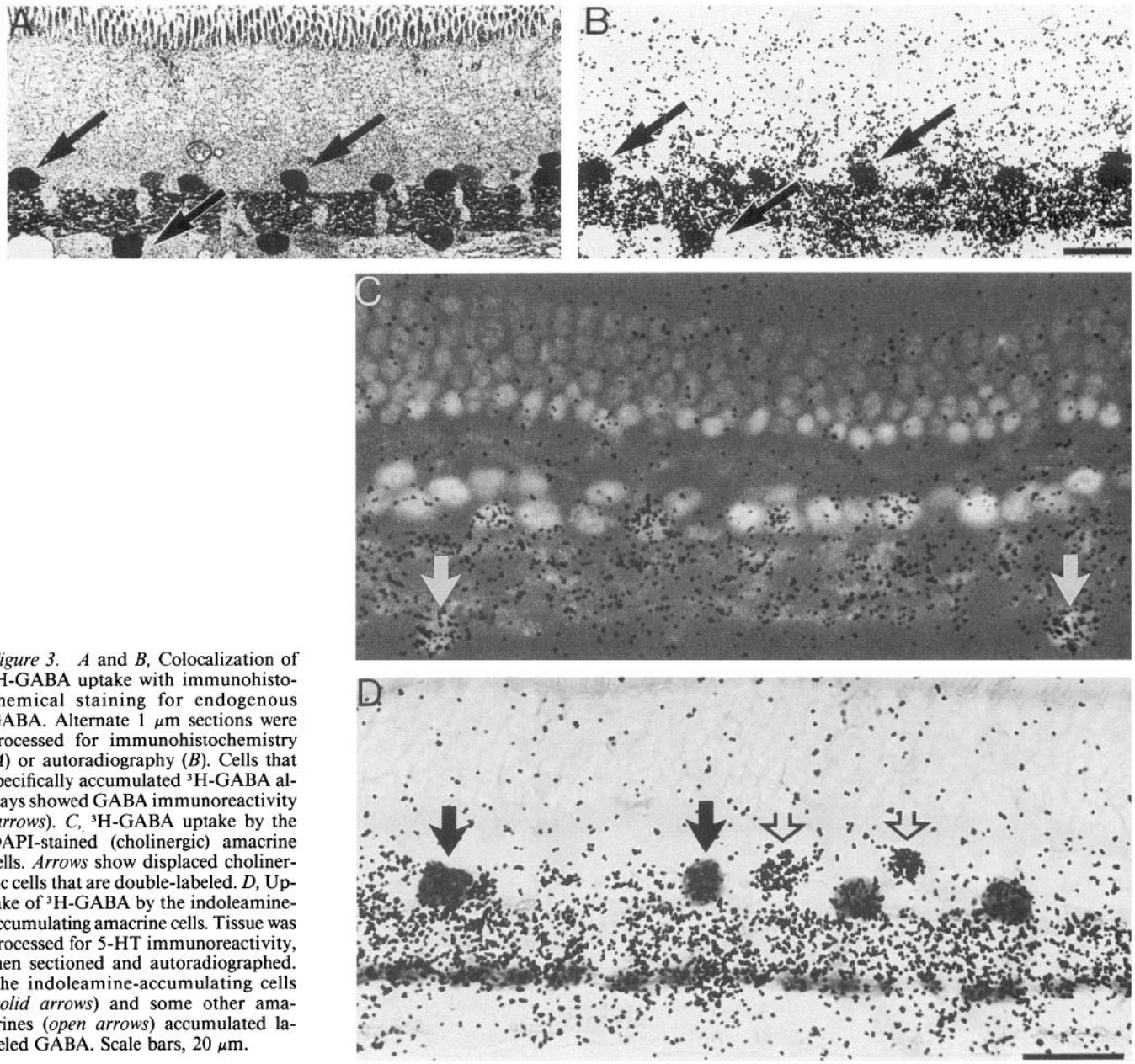

Figure 3. *A* and *B*, Colocalization of ^{3}H-GABA uptake with immunohistochemical staining for endogenous GABA. Alternate 1 μm sections were processed for immunohistochemistry (*A*) or autoradiography (*B*). Cells that specifically accumulated ^{3}H-GABA always showed GABA immunoreactivity (*arrows*). *C*, ^{3}H-GABA uptake by the DAPI-stained (cholinergic) amacrine cells. *Arrows* show displaced cholinergic cells that are double-labeled. *D*, Uptake of ^{3}H-GABA by the indoleamine-accumulating amacrine cells. Tissue was processed for 5-HT immunoreactivity, then sectioned and autoradiographed. The indoleamine-accumulating cells (*solid arrows*) and some other amacrines (*open arrows*) accumulated labeled GABA. Scale bars, 20 μm.

retinas were subsequently isolated and incubated in ^{3}H-GABA. The retinas were fixed in 4% paraformaldehyde to preserve the DAPI fluorescence (Fig. 3*C*). Although the autoradiographic localization of GABA is less sharp than in glutaraldehyde-fixed material, it is clear that the brightly fluorescent amacrine cell bodies in the ganglion cell layer, which correspond to displaced cholinergic amacrine cells, showed an accumulation of silver grains due to uptake of ^{3}H-GABA (arrows). In the inner nuclear layer, many DAPI-labeled (and some nonfluorescent) cells accumulated ^{3}H-GABA. These findings confirm that ^{3}H-GABA in our experiments was in fact contained in the starburst amacrine cells. The DAPI-positive cells are among those in the rabbit retina that are known to contain endogenous GABA (Brecha et al., 1988; Vaney and Young, 1988). Our results are also in accord with work carried out in the cat, where ^{3}H-muscimol uptake by amacrine cells was shown to correlate with the presence of ChAT (Chun et al., 1988).

In other experiments, rabbit retinas were incubated *in vitro* in the presence of 1 μM 5-HT, which was then detected immunohistochemically (Sandell and Masland, 1989a). The type I and type II indoleamine-accumulating cells showed clear accumulation of ^{3}H-GABA (Fig. 3*D*, solid arrows). When type III indoleamine-accumulating cells were observed (they are rare), they were heavily labeled with ^{3}H-GABA (Sandell and Masland, 1989b). Tritiated GABA was also accumulated by amacrine cells that did not take up 5-HT (Fig. 3*D*, open arrows). Similar results have been reported by Osborne et al. (1986) and more quantitative observations have been reported by Massey et al. (1991). We conclude that the retinas studied in our superfusion experiments contained radioactive GABA in the starburst amacrine

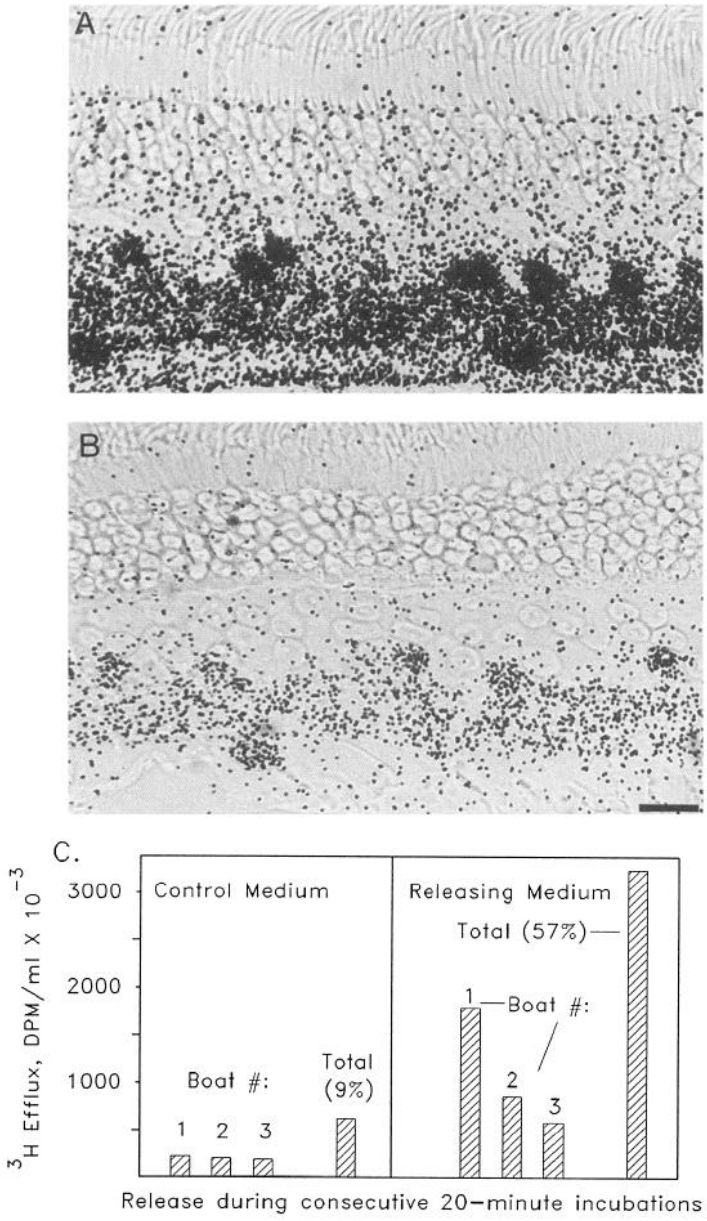

Figure 4. Release of ^{3}H-GABA from cholinergic amacrine cells. After labeling with ^{3}H-GABA, half-retinas were incubated in either control medium (*A*) or releasing medium (*B*). Each half-retina was transferred through three 6 ml boats for 20 min each. The two half-retinas were then fixed, processed together for autoradiography, and exposed for 10 d under identical conditions. Scale bar, 20 μm. *C,* The half-retina incubated in control medium released 9% of its initial ^{3}H-GABA content, while the half-retina incubated in releasing medium released 57% of its ^{3}H-GABA.

cells, the indoleamine-accumulating cells, and a population of other amacrine cells.

Release of GABA by the starburst cells

The release of GABA from specific cells in these experiments was observed autoradiographically. Retinas were labeled with ^{3}H-GABA for 1 hr, rinsed briefly, and cut in half along the dorsal-ventral axis. The half-retinas were incubated in control medium or in a medium designed to promote GABA release (20 mM KCl, 1 mM unlabeled GABA). The amount of ^{3}H-GABA taken up by each retina was approximated by measuring the amount of radioactivity removed by the retina from the labeling boat. The amount released was determined by counting aliquots of the medium into which GABA release had occurred. Control retinas released 9% of their initial contents of ^{3}H-GABA, while retinas incubated in releasing medium released 57% (Fig. 4*C*). Retinas that had been incubated in releasing medium showed fewer silver grains over the inner plexiform layer and over all of the amacrine cell bodies (Fig. 4*A,B*). The displaced cholinergic amacrine cells (easily identified because they represent most of the GABA-accumulating cells in the ganglion cell layer) were depleted of silver grains to the same extent as the population of GABA-accumulating cells as a whole. The cholinergic cells thus are shown to release GABA.

Effects of unbalancing the ON and OFF cell populations

Since several types of cells contain GABA, it is imaginable that the different cells' releases of GABA in response to photic stimulation are counterbalanced, such that an ON release by some cells is exactly matched by an inhibition of release in others, yielding no net outflow of GABA from the whole tissue. Superfused retinas were stimulated with flashing or moving lights in the absence or the presence of 4-amino-2-phosphonobutyric acid (APB), a compound that selectively eliminates ON responses from the inner retina (Slaughter and Miller, 1981, 1985; Massey and Redburn, 1983; Bloomfield and Dowling, 1985; Bloomfield and Miller, 1986). As expected, APB decreased the light-stimulated release of ACh (Neal et al., 1981; Masland et al., 1984b; Massey and Redburn, 1985). Despite elimination of the retina's ON responses, no light-modulated release of GABA was observed (Fig. 5). In fact, the diversity of the GABA-containing amacrine cells makes the failure of light to affect GABA release even more perplexing, since one or the other of the cells should surely be expected to respond.

Release of endogenously synthesized GABA

Retinas were incubated in the presence of ^{14}C-glutamate for 60 min, at the end of which time they had synthesized ^{14}C-GABA. The same retinas were simultaneously incubated in ^{3}H-GABA, which was accumulated by the cells in the usual way. They thus contained a mixture of ^{14}C-GABA synthesized from glutamate and ^{3}H-GABA accumulated from the medium. They were then incubated in control medium for 1 hr and releasing medium for a second hour. The amounts of ^{3}H-GABA and ^{14}C-GABA released were determined by electrophoresis of aliquots of the incubation boats. Potassium evoked equally large releases of endogenously synthesized ^{14}C-GABA and accumulated ^{3}H-GABA. Lowering the concentration of calcium in the medium did not inhibit the release of either isotopic form of GABA (Fig. 6).

Alternative methods of blocking reuptake

As was shown above (Fig. 1), nipecotic acid at ordinary concentrations gave evidence of preventing GABA reuptake but did not reveal a light-stimulated release of GABA. As will be shown below (see Fig. 12), this was also true even at concentrations up to 1 mM—orders of magnitude above those usually expected to block GABA reuptake. However, nipecotic acid gave evidence of being a substrate for the GABA carrier as well as competing for binding to it. We therefore studied the effect

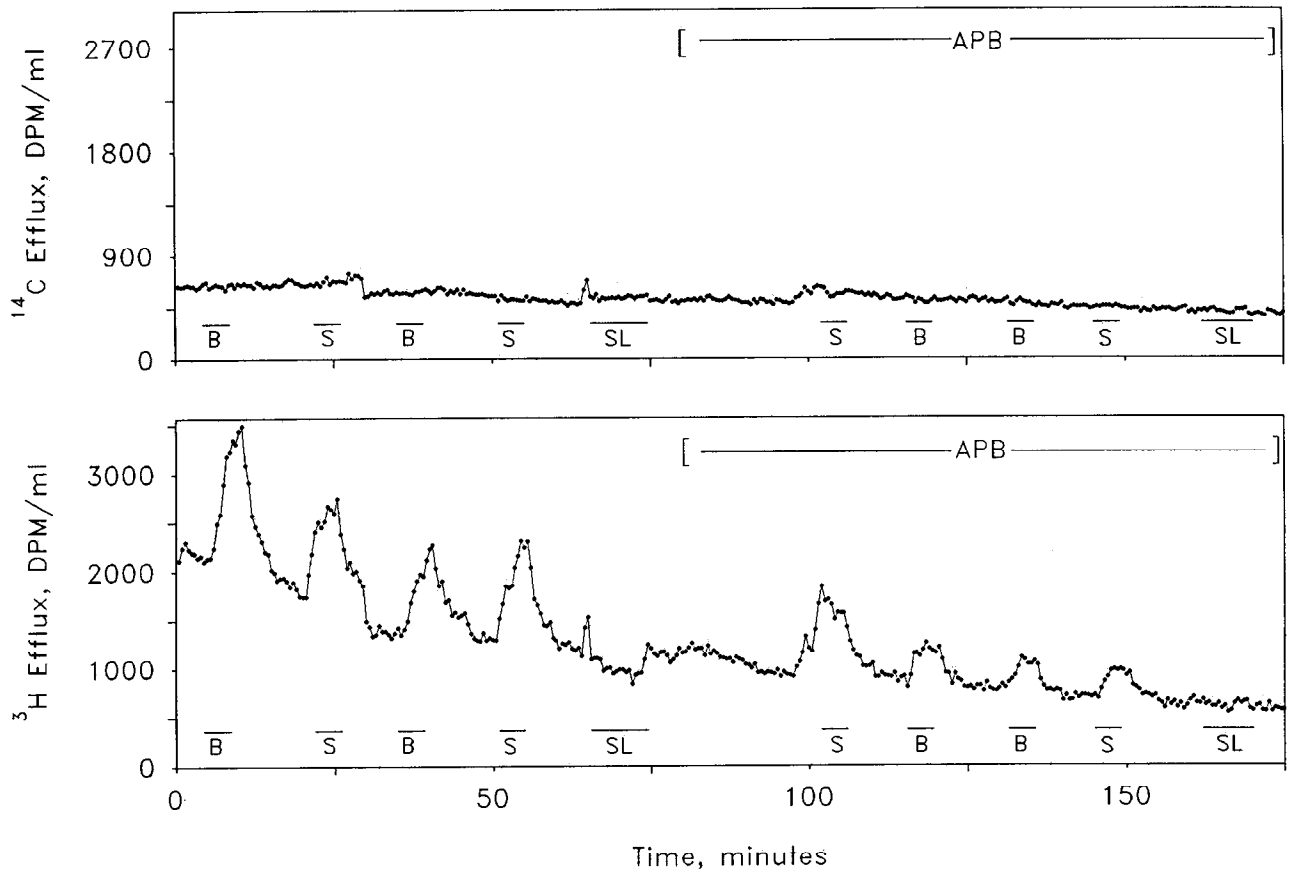

Figure 5. Effects of "ON" channel blockade upon the releases of ^{14}C-GABA (*top*) and ^{3}H-ACh (bottom). Retina was stimulated by 800 μm bars moving at 1600 μm/sec (*B*), strobe (*S*), or steady light (*SL*). APB at 30 μM was present as indicated.

of SKF 89976-A, a nonsubstrate antagonist of GABA uptake (Yunger et al., 1984). As for nipecotic acid, SKF 89976-A caused an increase in the recovery of GABA from the retina (Fig. 7). It failed to permit a light-stimulated release of GABA.

The increased concentration of GABA present in the extracellular space during reuptake blockade was large enough to have an effect on the activity of the inner retina. In fact, the rise in GABA concentration depressed the release of ACh during the pharmacological treatments—in accord with previous evidence for a strong, GABA-mediated inhibition of ACh release (Neal and Massey, 1980; Massey and Redburn, 1982). It confirms that the GABA reuptake blockers were effective in inhibiting reuptake. Conceivably, though, the rise in GABA could depress the inner retina's activity enough to suppress the release of GABA itself. We therefore exposed the retina first to nipecotic acid and later to nipecotic acid plus picrotoxin—the latter to release the retinal cells from inhibition caused by the artificially elevated extracellular GABA concentration. Figure 12 (below) will show that picrotoxin restored the retina's responsiveness, as indicated by the recovery of the light-stimulated release of ACh, but there was no light-evoked release of GABA (see Fig. 12).

As a test that avoids the use of pharmacological agents, we blocked reuptake by applying a high concentration of unlabeled GABA. It competed with radioactive GABA for reuptake and increased the recovery of radioactive GABA from the tissue, but never revealed a light-stimulated release of radioactive GABA (Fig. 8).

Manipulations of GABA metabolism

We studied the biochemical identity of the radioactive compounds present in the retina after GABA was accumulated from the extracellular space. In the experiments shown in Figure 9*A*,

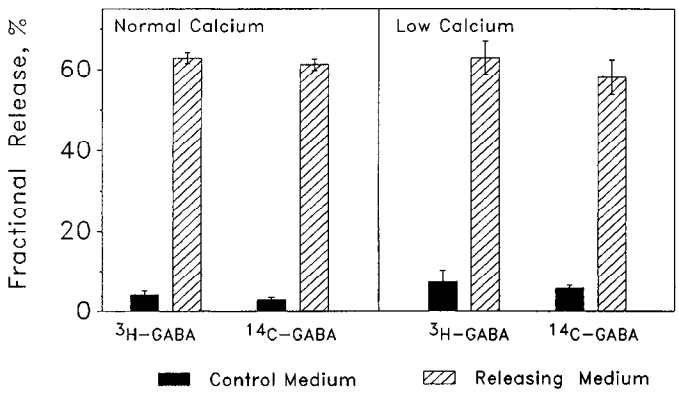

Figure 6. Release of endogenously synthesized GABA and GABA accumulated from the medium. Retinas were incubated in ^{3}H-GABA and ^{14}C-glutamate. Metabolism of GABA had been inhibited to allow accumulation of the ^{14}C-label into ^{14}C-GABA. After labeling, the retinas were rinsed briefly, incubated in 20 ml boats for 1 hr in control medium and then incubated for a second hour in releasing medium (20 mM potassium, 1 mM unlabeled GABA). The amount of ^{3}H-GABA and ^{14}C-GABA released into each boat was determined by electrophoresis. Data are means and SEs for four retinas.

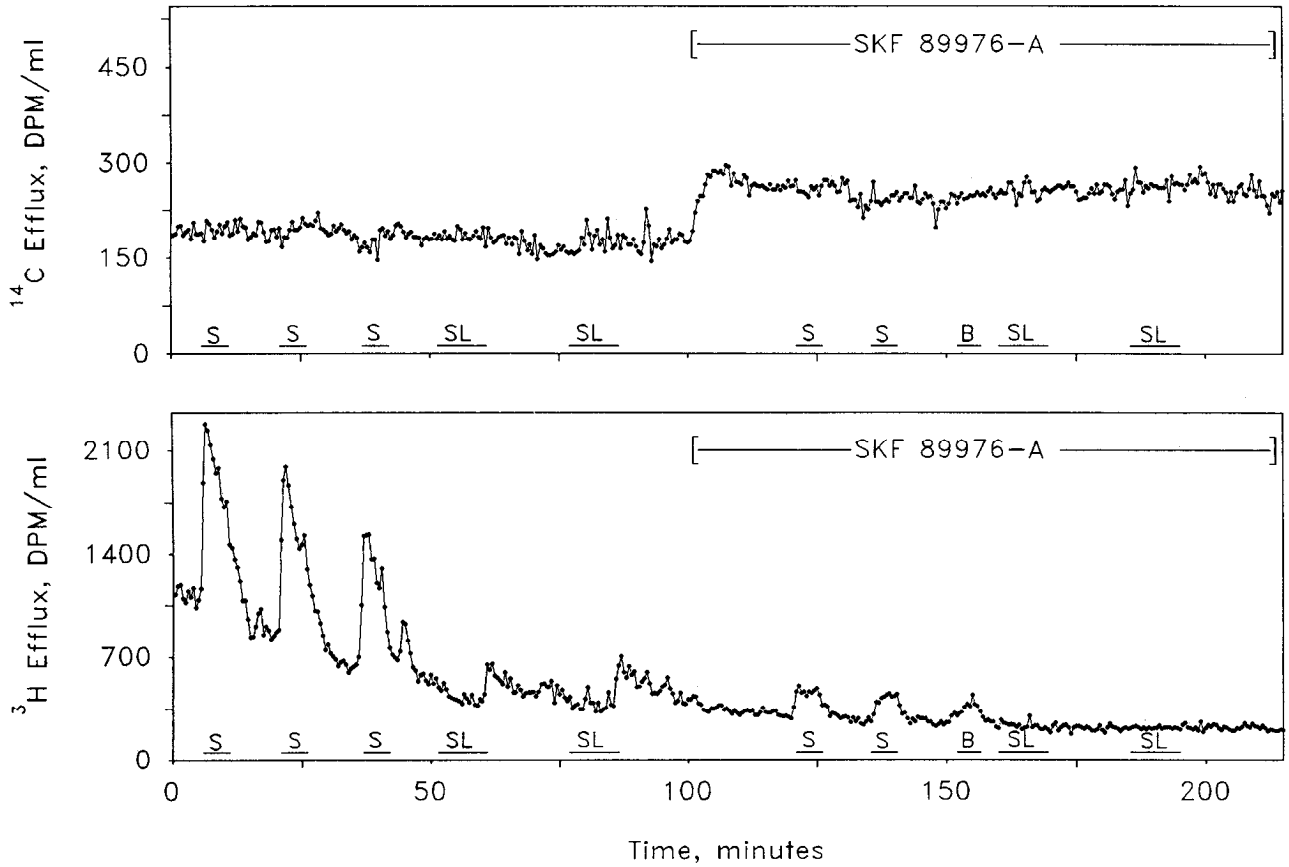

Figure 7. Effects of SKF 89976-A, a nonsubstrate blocker of GABA reuptake. Retina was stimulated by strobe (*S*), steady light (*SL*), or moving bars (*B*). In each pair of stimulations with steady light, the intensity of the first stimulation was 0.6 relative log units, and that of the second, 2.0 log units. SKF 89976-A at 30 μM was present where indicated. It caused an increased efflux of ^{14}C-GABA (*top*) and diminished the light-evoked release of ACh (*bottom*). (Steady light caused a slight inhibition of ACh release with a subsequent rebound.)

retinas were incubated in radioactive GABA for 1 hr and then briefly rinsed and homogenized or processed for autoradiography. The tissue was found upon electrophoresis to contain much radioactive GABA and peaks representing several GABA metabolites. The autoradiographic image (Fig. 9*A*, right) is dominated by labeled amacrine cells. In the experiments illustrated by Figure 9*B*, the retinas were incubated in the same way but with GABA-transaminase (GABA-T) inhibited by GVG. They

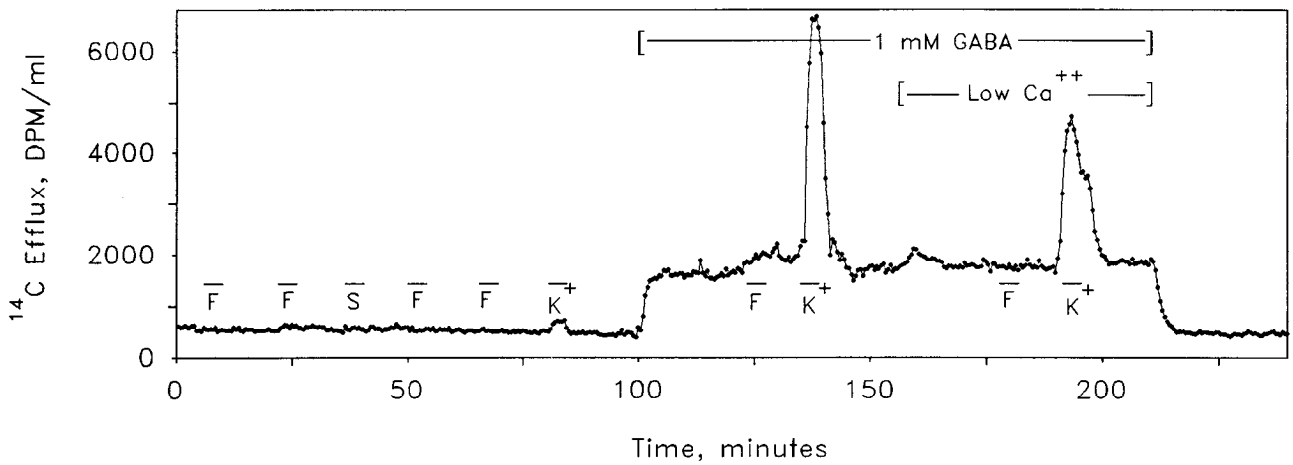

Figure 8. Effect of unlabeled GABA. The retina was stimulated by 50 msec light flashes at 2 Hz at two different intensities. The first was 0.6 relative log units; all subsequent ones were at 2.8. *F* indicates flashes; K^+ indicates 30 mM K^+.

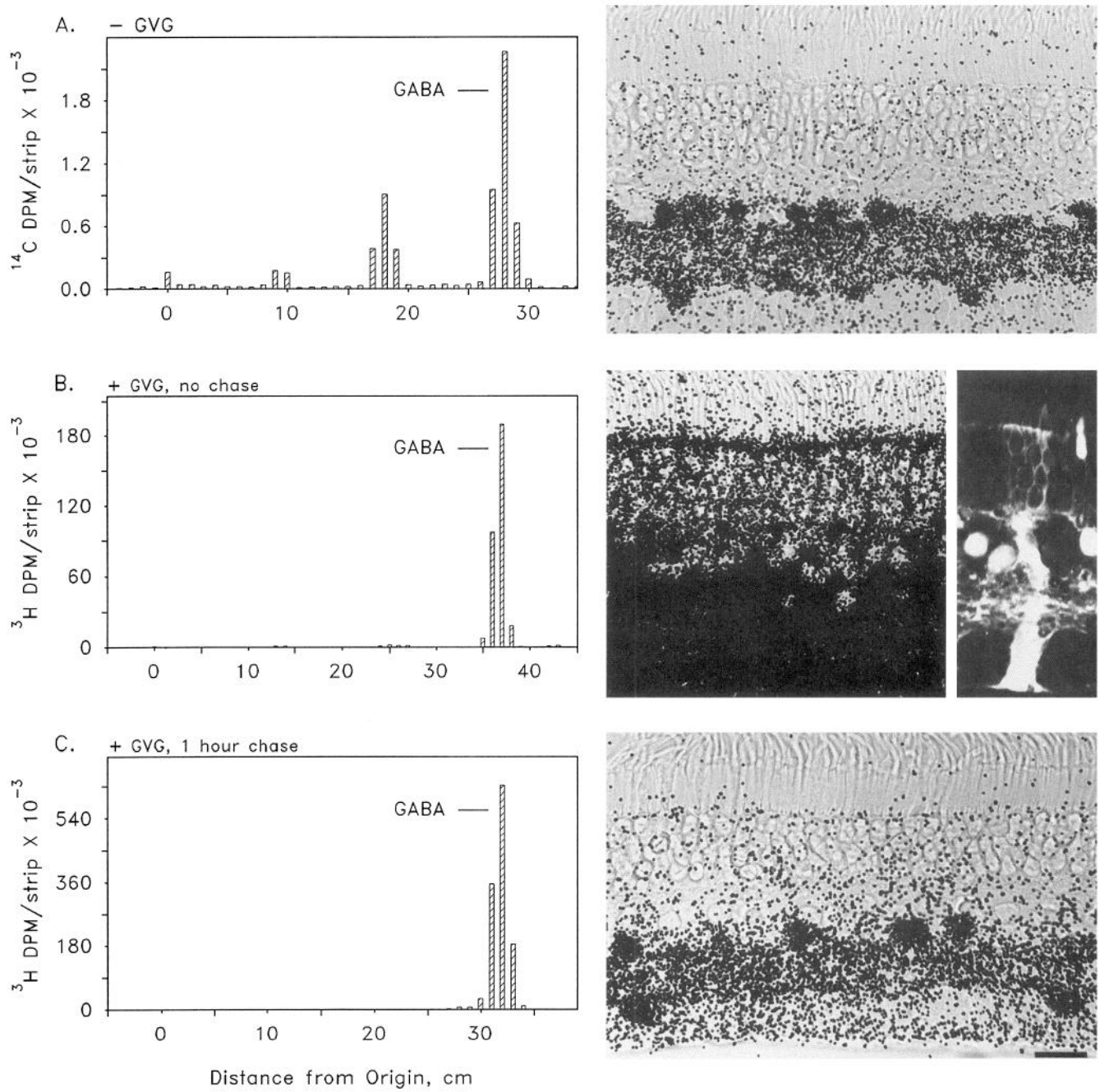

Figure 9. Effects of inhibiting GABA-T on the metabolism and anatomical distribution of GABA. *A,* Radiochemical content and autoradiograph of retinas labeled with radioactive GABA for 1 hr in medium containing no pharmacological agents. The tissue contains much GABA (*left*), but also contains non-GABA radioactivity, mainly in two peaks that migrated more slowly than GABA. The latter peaks appear to represent metabolites of GABA, since they only appeared when GABA-T was not inhibited; their identities were not studied further. *B,* Retina immediately after labeling by incubation for 1 hr in the presence of GVG. *Inset,* Müller cell filled with Lucifer yellow. *C,* Retina labeled for 1 hr in the presence of GVG then superfused for 1 hr in control medium. The tissue's radiochemical content is virtually all in the form of GABA (*left*), but GABA has been cleared from the Müller cells and is contained primarily in the neurons. Slight differences in the location of the peaks are due to slightly differing electrophoresis times. For rationale for usage of ^{14}C and ^{3}H, see Materials and Methods. Scale bar, 20 μm.

were rinsed and homogenized immediately after the exposure to labeled GABA. Electrophoretic separation showed virtually all of the tissue's radioactivity to be in the form of GABA. Autoradiography shows labeling of amacrine cells, but also a dense labeling that has the distribution of the Müller cells (Hyde and Robinson, 1974). The shape and position of Müller cells are indicated by the Lucifer-filled Müller cell inset in the photomicrograph. There is heavy labeling of the Müller end feet [which in the rabbit make up most of the volume of the ganglion cell layer (Robinson and Dreher, 1990)].

Although GABA initially accumulated in Müller cells when GABA-T was inhibited, the cells did not retain GABA indefi-

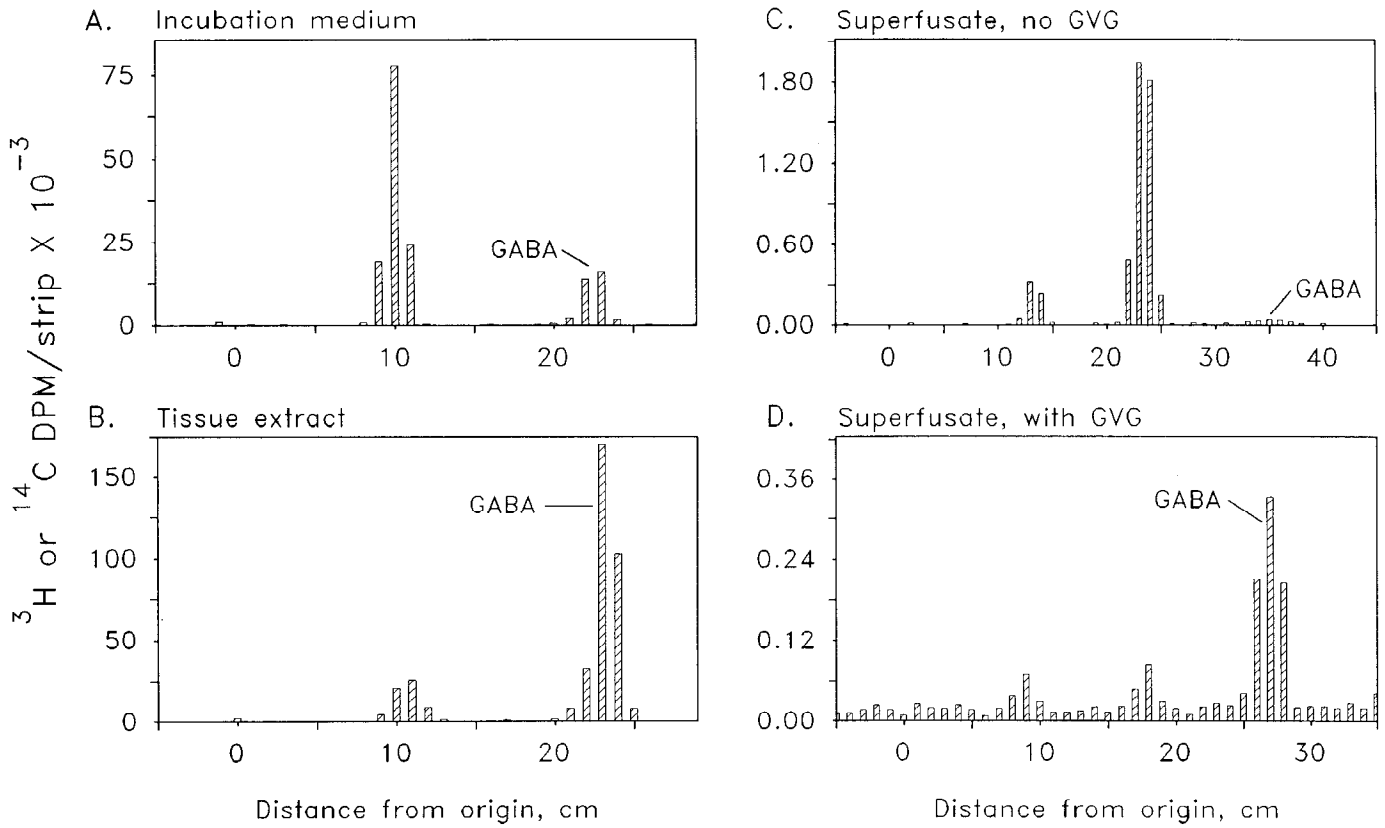

Figure 10. Analysis of radioactivity present in the tissue and incubation medium under several conditions. *A,* The radioactive compounds present in the labeling vessel after a retina had been in it for 1 hr. At the beginning of the incubation, the vessel's only radioactive compound was ^{3}H-GABA. The contents of the same retina at the end of incubation are shown in *B. B,* The tissue has accumulated a large pool of ^{3}H-GABA and retained it. *C,* Compounds released during an experiment in which the retina was labeled for 1 hr in the presence of ^{14}C-GABA (without GVG treatment) and then transferred to a superfusion chamber. *D,* Superfusate from a retina labeled with ^{14}C-GABA in the presence of GVG. The predominant compound released was ^{14}C-GABA.

nitely under those conditions. Figure 9*C* shows an experiment in which the retina was incubated with radioactive GABA as in Figure 9*B,* then transferred to control medium for 1 hr. Electrophoresis showed radioactive GABA as the tissue's main radioactive compound, but most of the labeled GABA was located in the amacrine cells. The GABA that builds up in Müller cells when GABA-T is inhibited is presumably cleared partly by release via the same carrier on which it originally entered. There is also evidence (see below) for a low level of GABA-T activity even after treatment with GVG; it would contribute additionally to clearing of labeled GABA from the Müller cells. Of practical importance is the finding that even in the presence of GVG, GABA is eventually contained mainly in the neurons. This means that GABA released from either normal retinas or GVG-treated retinas originated (when time was allowed for Müller cell clearance) from the amacrine cells.

We also studied the identity of the radioactive compounds released from the retina under various conditions. When normal retinas were incubated with ^{14}C-GABA, rinsed, and analyzed, most of the total radioactivity was found to have remained in the form of ^{14}C-GABA (Fig. 10*B*). However, only a few percent of the total radioactivity released into the superfusate was in the form of ^{14}C-GABA; most appeared as radioactive metabolites (Fig. 10*C*). When the retina was preincubated with GVG to inhibit GABA transaminase, metabolism of GABA was suppressed and 52.7 ± 3.6% of the ^{14}C-efflux from the retina was ^{14}C-GABA (mean ± SEM; n = 7). Stimulation with light released ACh from both GVG-treated and untreated retinas, but there was never a light-stimulated release of ^{14}C-labeled compounds—either as GABA or as GABA metabolites (Fig. 11*A,B*).

The fraction of the ^{14}C-efflux that was in the form of GABA was further increased when nipecotic acid was present in the superfusion medium. In GVG-treated retinas the fraction was 68.1 ± 2.9% in 0.1 mM nipecotic acid and 84.9 ± 0.5% in 1.0 mM nipecotic acid (n = 3). During stimulation with potassium, most of the increased ^{14}C efflux was due to an increased outflow of ^{14}C-GABA. These fractions did not change during photic stimulation.

The recovery of intact ^{14}C-GABA could alternatively be increased by blockade of GABA uptake in otherwise untreated retinas. As before, these treatments depressed the release of ACh, confirming the buildup of authentic GABA in the extracellular space. Figure 11*C* shows the effects of 30 μM SKF 89976-A. When unlabeled GABA or SKF 89976-A were present (but not in their absence), potassium caused a clear increase in the ^{14}C-efflux, which was mostly accounted for by an increased release of ^{14}C-GABA (Fig. 11*C,D*). Stimulation with light did not affect the ^{14}C efflux. The presence or absence of blockers of

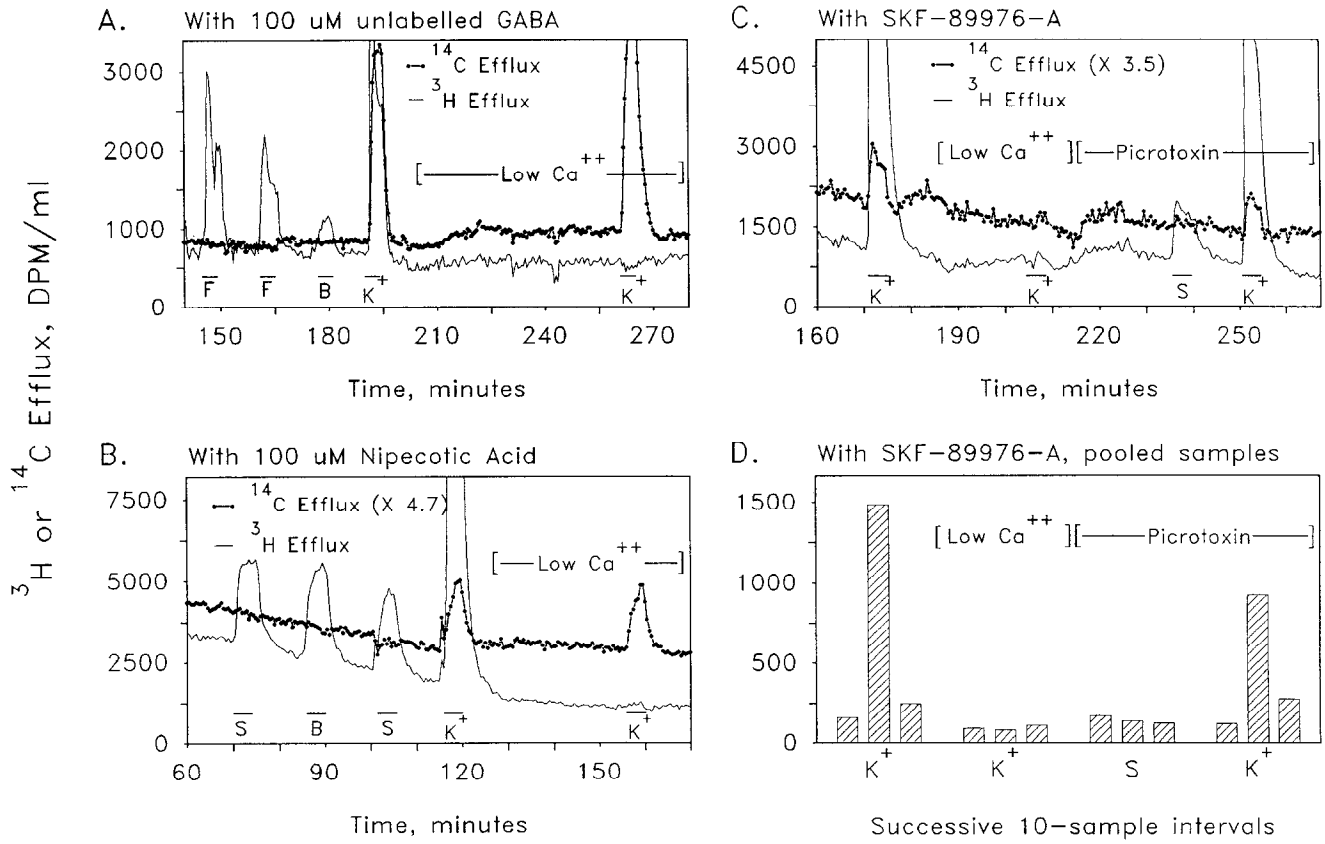

Figure 11. Potassium-stimulated release of ACh and GABA: effects of GABA-T inhibition and inhibiting reuptake of GABA. The ^{14}C-efflux traces in *B* and *C* have been scaled as indicated to allow ^{14}C and ^{3}H data to be plotted together. *A*, With 100 μM unlabeled GABA present, 30 mM potassium evoked large increases in ^{14}C-GABA efflux in normal or low-calcium medium. In these experiments, GABA-T was inhibited by GVG; approximately 70% of the basal ^{14}C-efflux and 95% of the K^+-evoked ^{14}C efflux is ^{14}C-GABA. Picrotoxin at 20 μM was present throughout. *B*, Nipecotic acid at 100 μM was used to block reuptake. Potassium at 20 mM evoked a smaller release of GABA that persisted in low-calcium medium. *C*, Potassium stimulation during blockade of GABA reuptake by SKF 89976-A. Retina not treated with GVG. Stimulation with 30 mM potassium evoked a large release of ^{3}H from ACh and a small increase in the total ^{14}C efflux from GABA, but K^+ had no effect on ^{14}C efflux in low Ca^{2+}. (In the presence of 30 μM SKF 89976-A, the fraction of the ^{14}C efflux that was ^{14}C-GABA increased from 2% to about 5%.) *D*, Groups of 10 samples from the superfusate in *C* were pooled immediately before, during, and after depolarization of the cells by 30 mM K^+. Their GABA was concentrated and ^{14}C-GABA was determined. In this case, the K^+-stimulated release of GABA was prevented by low-Ca^{2+} medium. When the retina was in medium containing normal concentrations of divalent cations, however, there was no release of ^{14}C-GABA in response to light (*S*, strobe flashes). The same photic stimulation evoked a clear release of ACh, as shown in *C*.

GABA reuptake had little effect on the potassium-evoked release of ACh. The light-evoked release of ACh was enhanced when picrotoxin was included in the medium. In no case was there a detectable photic modulation of ^{14}C efflux.

In the absence of GABA-T inhibition, most of the GABA released from the neurons was metabolized before release into the superfusate, as was noted above. In control medium and the unstimulated state less than 2% (1.29 ± 0.5; n = 16) of the ^{14}C efflux was ^{14}C-GABA. In the presence of 30 μM SKF 89976-A, the fraction increased to about 5% (5.28 ± 1.75; n = 28). There was no detectable modulation of the release of ^{14}C-GABA by photic stimuli (Fig. 11*C*). In this condition, there was evidence of a Ca^{2+} dependence of the K^+-stimulated GABA release. Because the amount of GABA released was small, we pooled samples from the superfusate and concentrated them (see Materials and Methods). When SKF 89976-A was included in the incubation medium, 30 mM potassium evoked a release of GABA that was abolished by low-calcium medium. Thus, three conditions were necessary for this calcium-dependent component of GABA release to be observed: (1) metabolism of GABA had to be uninhibited, (2) a nonsubstrate blocker of the transporter had to be present, and (3) the retina had to be stimulated by elevated K^+. We did not observe a light-evoked release of GABA under these conditions, despite 27 attempts using a wide range of photic stimuli.

Evidence for a carrier-mediated release of GABA

The K^+-induced release of GABA was, with the exception just mentioned, unaffected by exposure to medium containing 0.2 mM Ca^{2+} and 20 mM Mg^{2+}, while the stimulated release of ACh was entirely prevented. Independence of extracellular Ca^{2+} suggested that the K^+-stimulated release of GABA occurred by a nonvesicular mechanism. Further evidence was that recovery was increased by an excess of unlabeled GABA, traditional evidence of homoexchange. Nipecotic acid progressively increased GABA outflow at concentrations up to 10 mM (Fig. 12). This probably represents heteroexchange by the same carrier, since nipecotic acid appears to be a substrate for GABA carriers in

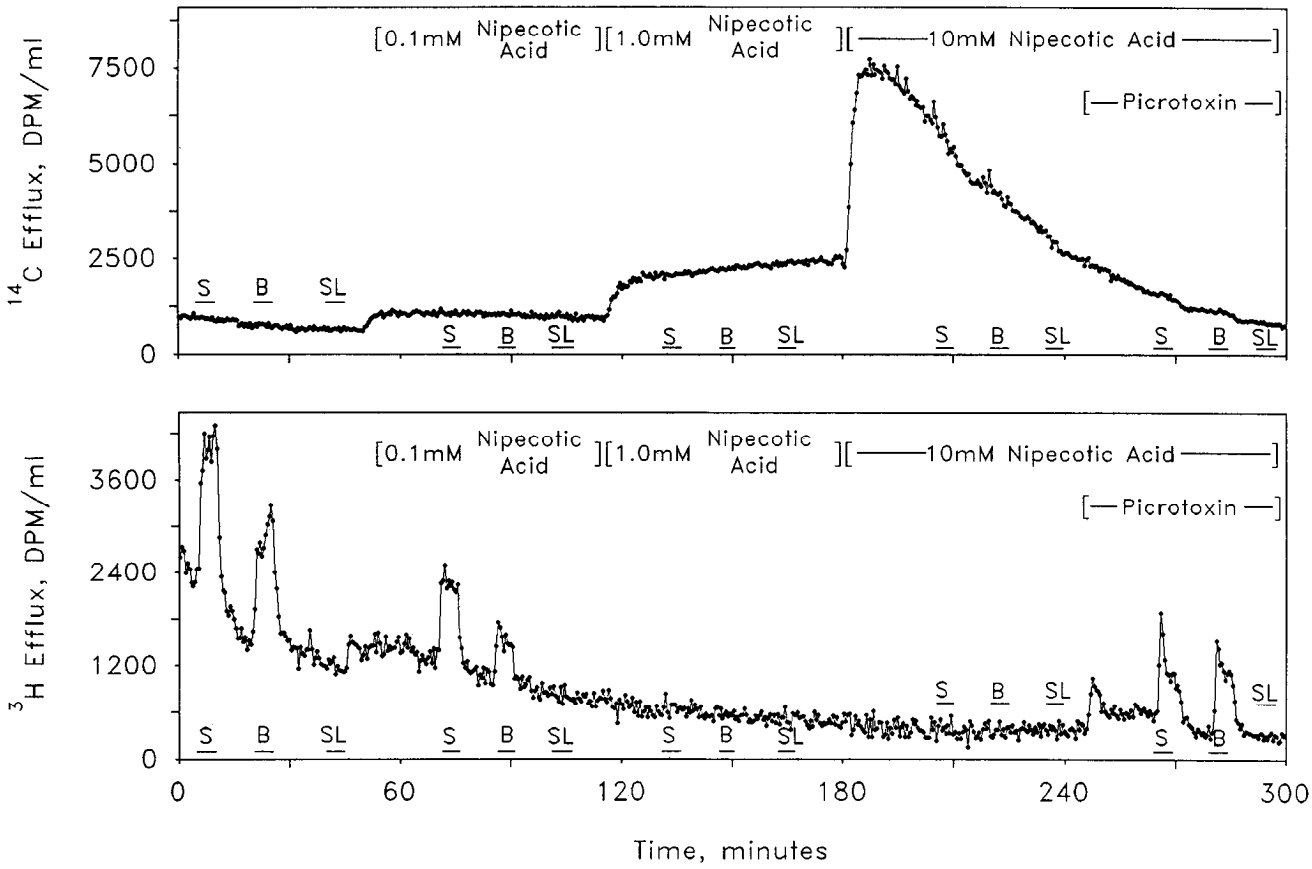

Figure 12. Effects of nipecotic acid on the release of ^{14}C from GABA and ^{3}H from ACh. The retina was stimulated by strobe flashes (*S*), moving bars (*B*), or steady light (*SL*) where indicated. All fractions were analyzed by scintillation counting. For selected fractions, 1 ml aliquots were pooled and analyzed for ^{14}C-GABA content. GABA-T was inhibited by GVG. The fractions of the ^{14}C efflux that was ^{14}C-GABA in the 10 fractions before, during, and after the first strobe stimulation were 53.3%, 49.9% and 46.1%, respectively; for the second strobe stimulation (in 0.1 mM nipecotic acid), the fractions were 67.4%, 71.2%, and 65.6%, and for the third strobe stimulation (in 1 mM nipecotic acid), 84.9%, 84.5% and 85.4%. Picrotoxin (20 μM) reversed the inhibition of ACh release caused by nipecotic acid, but did not affect GABA release.

the retina (Agardh and Ehinger, 1982). All of these were substantial effects, large enough that the buildup of GABA in the extracellular space often depressed the release of ACh.

Discussion

The overall system of GABA uptake and metabolism in the isolated rabbit retina behaved as would be expected from previous work. The amacrine cells shown by immunohistochemistry to contain endogenous GABA accumulated it from the medium. GABA was also accumulated by the Müller cells, where it was rapidly metabolized (Ehinger and Falck, 1971; Marshall and Voaden, 1975; Ehinger, 1977; Agardh et al., 1986, 1987; Redburn and Madtes, 1986; Mosinger and Yazulla, 1987; Wässle and Chun, 1989). The amacrine cells, in contrast, maintained a store of radiolabeled GABA for at least several hours. During this time, radiolabeled GABA contained in amacrine cells was the tissue's primary radiolabeled compound, and these were the conditions under which the release of GABA was studied. When the cells were depolarized by raising the extracellular concentration of K^+, the release of both ACh and GABA was increased. Autoradiography has previously shown that the starburst cells are the only cells in the rabbit retina to synthesize ACh from exogenous choline, and their depletion of radioactive ACh by stimulation has been directly demonstrated (Masland and Mills, 1979; Masland et al., 1984b). It seems clear that the starburst cells release both ACh and GABA.

However, the mechanisms of release had different requirements for extracellular Ca^{2+}. Lowering the extracellular concentration of Ca^{2+} (and raising Mg^{2+}) entirely prevented any stimulation of ACh release. Under most conditions, the release of GABA was Ca^{2+} independent, indicating that it did not occur by exocytosis of synaptic vesicles. Agents that compete for the GABA carrier (nipecotic acid, SKF 89976A, unlabeled GABA) caused large increases in the recovery of GABA. Nipecotic acid and unlabeled GABA caused concentration-dependent counterfluxes of GABA, and the counterfluxes continued to increase as extracellular substrate concentrations were raised to the millimolar range. This is evidence of vigorous homoexchange by the GABA carrier, and this suggests that a large portion of the GABA release observed under control conditions was by carrier.

That depolarization can move GABA outward on its carriers has been clearly demonstrated for retinal horizontal cells (Schwartz, 1982, 1987; Yazulla and Kleinschmidt, 1983). The carrier present on horizontal cells, like GABA carriers generally, cotransports Na^+ and is therefore electrogenic; outward movements of GABA are induced by decreases in the cell's membrane potential. Release of GABA from amacrine cells is strongly and immediately increased by ouabain, consistent with this mech-

anism (Agardh and Bauer, 1984). Carrier-mediated efflux induced by raising extracellular K^+ thus seems to account for most of the increased outflow of GABA observed when K^+ was raised in our experiments.

No light-induced release of GABA was observed, despite several hundred attempts under a wide variety of physiological and pharmacological conditions. This was puzzling, because the double-labeling procedure allows the evoked release of ACh to serve as an internal control. For example, the choice of photic stimuli is hard to blame. Those stimuli were effective in causing the starburst cells to release ACh. Since GABA is contained in the same cells, GABA should have been released as well.

It seems unlikely that this result is attributable to reuptake of the released GABA. Although the system for inactivation of released GABA is different from that for ACh (reuptake vs. hydrolysis), the difference in this context is less than it might seem. Choline produced by hydrolysis of ACh is subject to reuptake just as GABA is, yet a light-stimulated release of choline was readily observed. High concentrations of nipecotic acid, SKF 89976A, or unlabeled GABA failed to unmask a light-stimulated release of GABA, despite evidence that they caused a major interference with GABA reuptake. When inhibition of GABA-T was combined with reuptake blockade, the fraction of radioactivity released as GABA rose to as much as 85% of the total radioactivity released, yet there was still no light-stimulated component of the release. Since a significant fraction of the GABA released by the neurons diffused into the superfusing medium in the presence of these inhibitors, it should have reflected the putative light-stimulated release—particularly since the high sampling frequency and relatively smooth baselines in these experiments would have allowed detection of a light-stimulated effect of a few percent above baseline.

Finally, the release of metabolites of GABA would also be expected to reflect any light-stimulated release of GABA. As was shown by the autoradiographic experiments, the Müller cells rapidly metabolize the GABA that they take up, and release radiolabeled metabolites into the medium. In experiments where Müller cell uptake and metabolism were allowed to proceed normally, release of GABA from neurons by light should have resulted, after a short delay, in an increased efflux of GABA metabolites from the Müller cells. Such an effect was not observed.

In view of these findings, one must entertain the possibility that stimulation with light does not cause a release of GABA from amacrine cell synapses. From other evidence, though, lack of release of GABA seems extremely unlikely. GABA has strong effects on the inner retina's electrical activity (Caldwell and Daw, 1978; Caldwell et al., 1978; Bolz et al., 1985). Synapses of the GABA-containing amacrine cells have the usual aggregations of clear vesicles (Famiglietti, 1983; Chun and Wässle, 1989; Sandell et al., 1989), and the release of GABA in our experiments was not entirely Ca^{2+} independent. When the retina was treated with SKF 89976A and no GVG, some Ca^{2+} dependence was observed, and a recent preliminary report by Neal et al. (1991) describes a Ca^{2+}-dependent component for the release of endogenous GABA in the presence of GVG. A Ca^{2+}-dependent component was also described in measurements of GABA release from retinal synaptosomes (Redburn et al., 1976; Redburn, 1977).

When synaptic vesicles were precipitated from double-labeled retinas, ACh coprecipitated with vesicles but GABA did not (O'Malley and Masland, 1989). However, amino acid–containing vesicles appear to concentrate their neurotransmitters by different mechanisms than cholinergic vesicles (Hell et al., 1988, 1990; Maycox et al., 1990), and the precipitation experiment is not definitive. What does seem clear is that the amount of GABA stored in the cytoplasm of many neurons is large (Nicholls, 1989; Maycox et al., 1990). This would provide a large pool of GABA for carrier-mediated release.

A likely interpretation of our results is that the carrier-mediated release is so large that it obscures whatever synaptic (vesicular) release may occur. However, two difficulties with this argument must be resolved. First, if the carrier mediates the increased GABA release that results when the extracellular concentration of K^+ is elevated, why does it not do so when the cells are depolarized by stimulation of the retina with light? This problem can be met by postulating that the membrane potential modulates carrier-mediated GABA release quite shallowly, so that the small (and brief) depolarizations caused by photic stimulation do not cause much GABA release compared to the large, prolonged depolarizations produced by K^+. The second problem is that the same K^+ depolarizations did trigger a Ca^{2+}-dependent release of ACh, presumably via cholinergic synaptic vesicles located in the starburst cells, while the K^+-stimulated release of GABA from these cells remained largely Ca^{2+} independent. Elevating K^+ thus appears to have caused the presynaptic events necessary for secretion via synaptic vesicles (at least those that contain ACh), without causing much Ca^{2+}-dependent GABA release. This problem can be met by assuming that the carrier-mediated release is modulated around a high resting rate—high enough to obscure the vesicular release of GABA. This would imply that the inner retina is subject at rest to a substantial carrier-mediated GABA "tone," a possibility consistent with the dramatic effects of GABA and its antagonists on the spontaneous activity of the retinal ganglion cells (Caldwell and Daw, 1978; Caldwell et al., 1978; Bolz et al., 1985).

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