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GABA transport and calcium dynamics in horizontal cells from the skate retina

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1. Changes in intracellular calcium concentration $[Ca^{2+}]_i$ in response to extracellularly applied γ-aminobutyric acid (GABA) were studied in isolated horizontal cells from the all-rod skate retina.

2. Calcium measurements were made using fura-2 AM, both with and without whole-cell voltage clamp. Superfusion with GABA, in the absence of voltage clamp, resulted in an increase in $[Ca^{2+}]_i$; the threshold for detection was approximately 50 μM GABA, and a maximal response was elicited by 500 μM GABA.

3. The rise in $[Ca^{2+}]_i$ was not mimicked by baclofen nor was it blocked by phaclofen, picrotoxin or bicuculline. However, the GABA-induced $[Ca^{2+}]_i$ increase was completely abolished when extracellular sodium was replaced with *N*-methyl-D-glucamine.

4. With the horizontal cell voltage clamped at −70 mV, GABA evoked a large inward current, but there was no concomitant change in $[Ca^{2+}]_i$. Nifedipine, which blocks L-type voltage-gated Ca^{2+} channels, suppressed the GABA-induced increase in $[Ca^{2+}]_i$. These findings suggest that the calcium response was initiated by GABA activation of sodium-dependent electrogenic transport, and that the resultant depolarization led to the opening of voltage-gated Ca^{2+} channels, and a rise in $[Ca^{2+}]_i$.

5. The GABA-induced influx of calcium appears not to have been the sole source of the calcium increase. The GABA-induced rise in $[Ca^{2+}]_i$ was reduced by dantrolene, indicating that internal Ca^{2+} stores contributed to the GABA-mediated Ca^{2+} response.

6. These observations demonstrate that activation of the GABA transporter induces changes in $[Ca^{2+}]_i$ which may have important implications for the functional properties of horizontal cells.

In the retina of many vertebrate species, γ-aminobutyric acid (GABA) is the inhibitory neurotransmitter of some classes of horizontal cells (cf. Yazulla, 1986). These post-receptoral neurons extend processes laterally within the outer plexiform (synaptic) layer of the distal retina where, through feedback to photoreceptors or feedforward interaction with bipolar cells (Wu, 1994), they exert a profound effect on the receptive-field organization and photic sensitivity of more proximal neurons in the visual pathway (Naka & Witkovsky, 1972). As with other amino acid neurotransmitters, the postsynaptic action of GABA is terminated by uptake into neurons and glia surrounding the release site (Ripps & Witkovsky, 1985; Yazulla, 1986). This complex machinery is still poorly understood, and it is not clear whether the electrogenic uptake of GABA affects other intracellular processes that may, in turn, influence intercellular communication.

One such event concerns the effect of the GABA transport mechanism on the concentration of intracellular calcium ($[Ca^{2+}]_i$), a ubiquitous second-messenger that modulates a wide range of intracellular processes, e.g. neurotransmitter release, Ca^{2+}-activated calcium release, and the junctional conductance between electrically coupled cells. Owing to some of its unique properties, the skate horizontal cell provides a useful model system in which to address this issue: there is evidence that GABA is the neurotransmitter utilized by these cells (Brunken, Witkovsky & Karten, 1986; Agardh, Bruun, Ehinger, Ekstrom, van Veen & Wu, 1987), large currents are generated by a sodium-dependent electrogenic GABA transport mechanism (Malchow & Ripps, 1990), isolated cells appear to be entirely free of GABA/benzodiazepine receptors (Malchow & Ripps, 1990), and skate horizontal cells are electrically coupled to their neighbours by gap junctions (Qian & Ripps, 1992; Qian,

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Malchow & Ripps, 1993). In the present study we examine the effects of GABA on the concentration of intracellular Ca^{2+}, the concomitant changes in membrane current and voltage, and the sources of the GABA-mediated changes in $[Ca^{2+}]_i$.

METHODS

Cell dissociation

The procedure for obtaining isolated horizontal cells has been described in detail previously (Malchow, Qian, Ripps & Dowling, 1990). Briefly, eyes were enucleated from skate (*Raja erinacea* and *Raja ocellata*) that had been sedated on ice, anaesthetized with 0·02% MS222 (tricaine), and pithed both anteriorly and posteriorly. After hemisecting the globe and removal of most of the vitreous humour, pieces of neural retina were excised from the posterior eyecup. The preparation was incubated for about 60 min in an L-15 culture medium (Sigma Chemical Co.) containing papain (2 mg ml^{-1}) and L-cysteine (1 mg ml^{-1}), and modified for use with skate by the addition of 103 mM NaCl, 350 mM urea, 5 mM glucose, and 20 mM Hepes, adjusted to pH 7·6 with NaOH. After incubation, the retinal pieces were rinsed 8–10 times with enzyme-free, skate-modified culture medium, and then triturated repeatedly (~50 times) through a large bore pipette. An aliquot of the supernatant containing isolated cells was placed on a glass coverslip that was sealed around a 15 mm aperture in the base of a plastic culture dish to form a shallow chamber. The coverslip was coated with protamine sulphate (1–10 mg ml^{-1}) for at least 2 h followed by concanavalin A (1 mg ml^{-1}) for 6 h, and rinsed thoroughly with distilled water prior to use. Cells were maintained in an incubator at 14 °C, and used 1–4 days after isolation.

During recordings, the cells were continuously superfused with solutions applied via a multibarrel pipette positioned about 500 μm from the cell surface; the flow rate was approximately 2 ml min^{-1}, and the perfusate was removed from the culture dish by suction. Solution changes were effected by a mechanically controlled multivalve system, with a delay time of about 2 s for fluid exchange. The skate Ringer solution contained (mM): 250 NaCl, 6 KCl, 5 $NaHCO_3$, 1 $MgCl_2$, 4 $CaCl_2$, 0·2 NaH_2PO_4, 360 urea, 10 glucose and 10 Hepes; adjusted to pH 7·6 with NaOH. Except for the sodium substitution experiments in which equimolar concentrations of *N*-methyl-D-glucamine (NMG) replaced sodium chloride (pH adjustment with HCl), and the calcium current measurements, in which barium replaced calcium, GABA-related compounds were added to the Ringer solution without modification.

Electrophysiology

Studies were conducted at room temperature (22–25 °C) with the dishes mounted on the stage of an inverted microscope and viewed under Nomarski optics; only the large external horizontal cells of the skate retina, readily identified by their characteristic morphology (Malchow *et al.* 1990), were used for this study. Membrane currents were monitored using the whole-cell version of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Pipettes with tip diameters of ~1 μm were pulled from Kovar capillary tubing (i.d. 1·15 mm, o.d. 1·65 mm, No. 7052; Garner Glass, Claremont, CA, USA), and used without flame polishing. The pipettes were filled with a solution consisting of (mM): 204 KCl, 2 $MgCl_2$, 2 MgATP and 10 Hepes (pH 7·6); and connected via a Ag–AgCl wire to the input stage of a Axopatch 200 patch-clamp amplifier (Axon Instruments). The output of the amplifier was stored on computer disks through a Labmaster A/D converter (Scientific Solutions, Solon, OH, USA), and data analysis was performed off-line with the pCLAMP software program (version 5.5; Axon Instruments).

$[Ca^{2+}]_i$ imaging

Fluorescence measurements were made using the membrane-permeable dye fura-2 AM (Molecular Probes, Inc.). A 50 μg aliquot of dye was reconstituted in 20 μl dimethyl sulphoxide (DMSO) that contained 20% (w/v) Pluronic F-127 detergent (Molecular Probes), and the mixture was added to 10 ml of Ringer solution to give a final concentration of 5 μM fura-2 AM, 0·2% DMSO and 0·04% Pluronic. Cells were incubated in the fura-2 AM solution in the dark for 30 min at 14 °C, rinsed with Ringer solution, and maintained for about 60 min to allow the acetoxymethyl groups to be cleaved from the dye. The culture dish was placed on the stage of a Zeiss Axiovert 35 epifluorescence microscope equipped with quartz optics (Carl Zeiss). The light from a 150 W xenon burner was passed alternately (rate of alternation approximately 2 Hz) through narrow-band interference filters of 334 and 380 nm to serve as the dual-wavelength source of excitation. Fluorescence emission was transmitted by a broad bandpass filter ($\lambda > 510$ nm), and monitored with an image-intensified CCD camera. The video signals were digitized to 8-bit resolution (256 shades of grey, 512 × 512 pixels per frame), and the emitted intensity at each of the two excitation wavelengths was recorded from within several user-defined intracellular areas. Typically, six to nine boxes (each ~10 × 10 pixels) were overlaid on the cell, and the averaged intensities within each box were recorded and analysed with the Attofluor digital microscopy system (Atto Instruments, Bethesda, MD, USA). No significant differences were observed between boxes at different loci; consequently, fluorescence measurements across the cell were averaged.

The intracellular Ca^{2+} concentration was determined from the fluorescence measurements using the equation (Grynkiewicz, Poenie & Tsien, 1985):

$$[Ca^{2+}] = K_d (R - R_{min})/(R_{max} - R) F_{380,zero}/F_{380,sat},$$

where K_d is the dissociation constant (450 nM) for fura-2 binding of Ca^{2+} (Williams & Fay, 1990), R is the ratio of fluorescent intensities measured at 334 and 380 nm (F_{334}/F_{380}), R_{min} is the ratio at zero Ca^{2+}, R_{max} is the ratio at saturated Ca^{2+}, and $F_{380,zero}$ and $F_{380,sat}$ are the fluorescence intensities resulting from excitation (at 380 nm) of the two solutions. The value of K_d in an intracellular environment is difficult to determine accurately, but an error in the estimate (essentially a scaling factor) would not affect the interpretation of the results. Before each experiment, ratio parameters were determined from fluorescence intensity measurements using standard solutions containing 10 μM fura-2, 10 mM EGTA, and either 0 Ca^{2+} or 10 mM Ca^{2+}, to provide zero Ca^{2+} and saturating Ca^{2+} (40 μM free Ca^{2+}) concentrations, respectively. Typical values were $R_{min} = 0·165$, $R_{max} = 3·89$ and $F_{380,zero}/F_{380,sat} = 7·47$. Background fluorescence, i.e. fluorescent signals from cells prior to dye loading, was not detectable in horizontal cells. Moreover, the use of dantrolene, a coloured compound with fluorescent properties, did not significantly affect the calcium-dependent signals.

RESULTS

GABA-induced changes in $[Ca^{2+}]_i$

Figure 1*A* shows changes in $[Ca^{2+}]_i$ derived from fluorescent intensity measurements (Fig. 1*B*) recorded alternately at 380 and 334 nm before, during and after 30 s applications of GABA at concentrations ranging from 10 to 500 μM; the corresponding changes in membrane potential recorded under current clamp (0 nA) are shown in Fig. 1*C*. The two nearly superimposed lower traces of Fig. 1*A* are representative of Ca^{2+} recordings obtained in response to 10 and 20 μM GABA. No change in $[Ca^{2+}]_i$ was detectable with 10 μM GABA (8 cells) or 20 μM GABA (2 cells), although both concentrations elicited small membrane depolarizations of 5–15 mV (Fig. 1*C*). However, higher concentrations of GABA, i.e. 50–500 μM, resulted in large increases in $[Ca^{2+}]_i$, and marked shifts in membrane potential. Several features of the responses are particularly noteworthy. First is the fact that with 50 and 100 μM GABA, the horizontal cell membrane potential rose to a level of about −50 mV, at which point it gave rise to a rapid depolarization that reached approximately −10 mV, almost identical to the initial depolarization induced by 500 μM GABA. The onset of these transients corresponded closely to the onset of the rise in $[Ca^{2+}]_i$, and the calcium increase tended to occur earlier as the GABA concentration was increased. Second, the more prolonged the depolarization, the greater was the magnitude of the increase in $[Ca^{2+}]_i$;

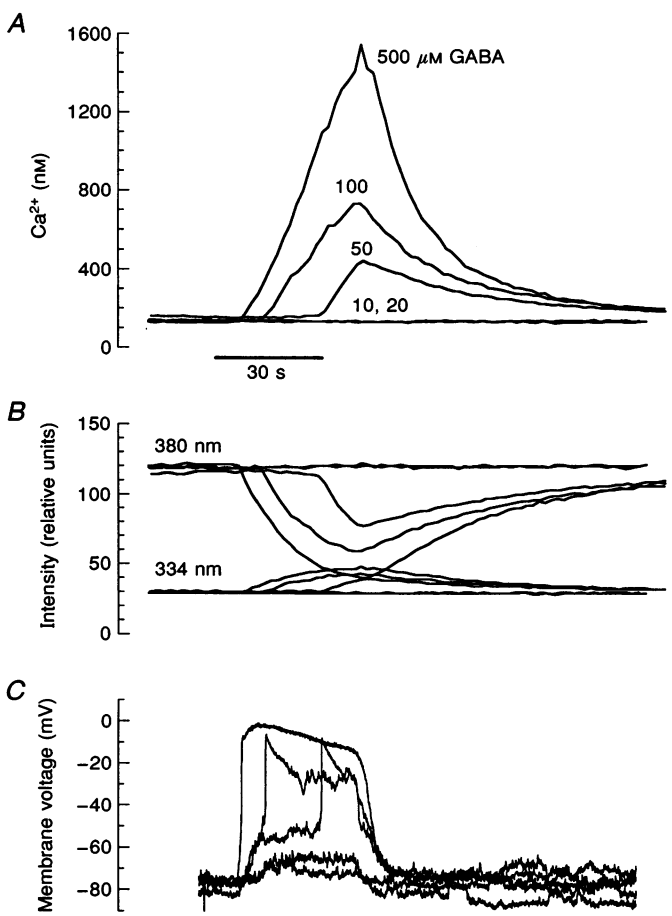

Figure 1. GABA-induced changes in $[Ca^{2+}]_i$ and membrane potential in a skate horizontal cell
Recordings were made under current clamp (0 nA). *A*, the intracellular $[Ca^{2+}]$ increase that resulted from the application of 10 to 500 μM GABA. Responses were measured in the same cell, and the time bar indicates when the superfusate was switched from Ringer to the GABA solution. *B*, fluorescence emission (relative units) measured alternately at 380 and 334 nm. See Methods for details. *C*, the corresponding membrane voltage responses measured from the same current-clamped cell. No change in $[Ca^{2+}]_i$ was induced by 10 or 20 μM GABA, and the membrane depolarization was small. For 50, 100 and 500 μM GABA, there was an increase in $[Ca^{2+}]_i$; higher concentrations of GABA (> 500 μM) did not produce a larger $[Ca^{2+}]_i$ increase (data not shown). The duration of the membrane depolarization (> −40 mV) increased as [GABA] was increased. Similar results were obtained from measurements on 8 cells.

in response to 500 μM GABA, intracellular calcium increased to 1·05 ± 0·53 μM from a mean resting level of 120 ± 45 nM (means ± S.D., $n = 56$). Finally it should be noted that $[Ca^{2+}]_i$ remained elevated above baseline long after the membrane potential had returned to its resting level. This phenomenon may be due, in part, to translocation of Ca^{2+} into intracellular stores, extrusion via an exchange mechanism, or Ca^{2+} buffering by endogenous intracellular buffers or by fura-2.

Pharmacology of the GABA-induced Ca^{2+} response

In attempting to identify the mechanism(s) mediating the GABA-induced rise in intracellular Ca^{2+}, various pharmacological agents were introduced in the bath solution. As shown in Fig. 2*A* and *B*, there is no indication that activation of $GABA_B$ receptors is involved in the process. The $GABA_B$ receptor agonist baclofen (500 μM), that in some cell types regulates a Ca^{2+} conductance (Slaughter & Pan, 1992), did not induce a change in the resting level of $[Ca^{2+}]_i$ (Fig. 2*A*), and the $GABA_B$ receptor antagonist phaclofen failed to suppress the GABA-induced Ca^{2+} increase (Fig. 2*B*). Similarly, $GABA_A$ receptors do not appear to play a role in mediating the Ca^{2+} response; the addition of 250 μM picrotoxin (Fig. 2*C*) or 250 μM bicuculline (Fig. 2*D*) to the GABA solution had no detectable effect on the GABA-evoked changes in $[Ca^{2+}]_i$.

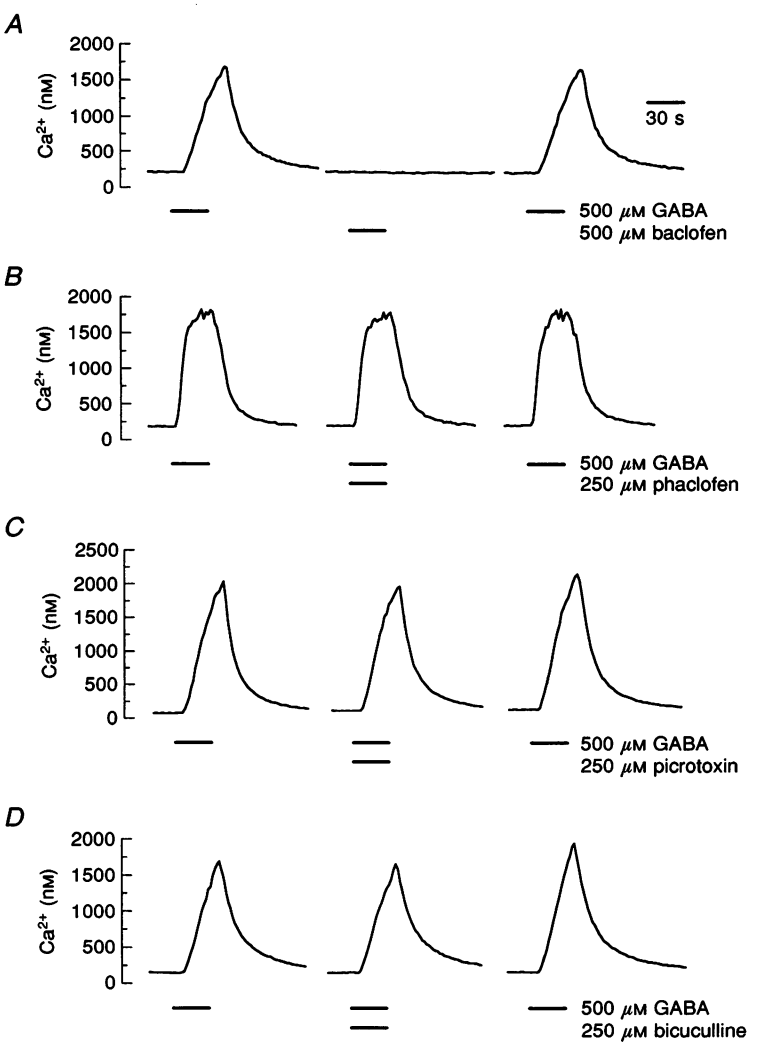

Figure 2. Pharmacology of the GABA-induced $[Ca^{2+}]_i$ response

A, the $[Ca^{2+}]_i$ increase in response to 500 μM GABA was not mimicked by 500 μM baclofen, a $GABA_B$ receptor agonist. *B*, the $[Ca^{2+}]_i$ increase in response to 500 μM GABA was not blocked by the addition of 250 μM phaclofen, a $GABA_B$ receptor antagonist. *C* and *D*, neither picrotoxin nor bicuculline (250 μM), $GABA_A$ receptor antagonists, had an effect on the rise in $[Ca^{2+}]_i$ induced by 500 μM GABA. Responses plotted on the same graph are from the same cell, and in each case, the experiments were repeated on at least 7 other cells with similar results.

GABA transport mediates the changes in $[Ca^{2+}]_i$

Skate horizontal cells possess a sodium-dependent GABA transporter that imports Na^+, Cl^- and GABA, resulting in a net inward current (Malchow & Ripps, 1990). The experimental results shown in Fig. 3 suggest that the electrogenic uptake of GABA, and the concomitant membrane depolarization, are key factors in triggering the Ca^{2+} response to exogenous GABA. The removal of Na^+ from the superfusate abolished completely the GABA-induced increase in $[Ca^{2+}]_i$ (Fig. 3*A*); furthermore, the current-clamp recordings of Fig. 3*B* show that removal of Na^+ also suppressed the membrane depolarization.

Further evidence that activation of the GABA transporter, and not $GABA_A$ or $GABA_B$ receptors, underlies both the GABA-mediated current response and the GABA effect on $[Ca^{2+}]_i$, is provided by the series of voltage-clamp recordings shown in Fig. 4. These experiments, in which membrane current and $[Ca^{2+}]_i$ were recorded simultaneously with the horizontal cell held at −70 mV, again show that picrotoxin and bicuculline, agents that block the activity of $GABA_A$ receptors, and phaclofen, the $GABA_B$ receptor blocker, had no direct effect on $[Ca^{2+}]_i$, nor did they alter the GABA-induced inward currents (Fig. 4*A–C*). On the other hand, the removal of sodium from the bath solution suppressed completely the inward current, although a small, slow rise in $[Ca^{2+}]_i$ was often observed under these conditions (Fig. 4*D*). Moreover, the voltage-clamp recordings of Fig. 4 demonstrate clearly the depolarization dependence of the Ca^{2+} response. By eliminating the voltage swing, i.e. with the horizontal cell held at −70 mV, the application of GABA produced inward currents of 400–750 pA, but no change in $[Ca^{2+}]_i$.

Mechanisms mediating the $[Ca^{2+}]_i$ increase

The failure to detect a GABA-induced change in $[Ca^{2+}]_i$ in cells voltage clamped at negative holding potentials suggests that the GABA-mediated changes shown in Fig. 1 are due primarily to calcium entry through voltage-gated Ca^{2+} channels (Malchow *et al.* 1990). If this were the case, membrane depolarization resulting from the electrogenic transport of GABA would lead to activation of a voltage-sensitive Ca^{2+} conductance (Malchow *et al.* 1990) that promotes, in turn, a rise in intracellular calcium. To test this possibility, GABA was applied to cells before and after superfusion with 100 μM nifedipine, a blocker of L-type

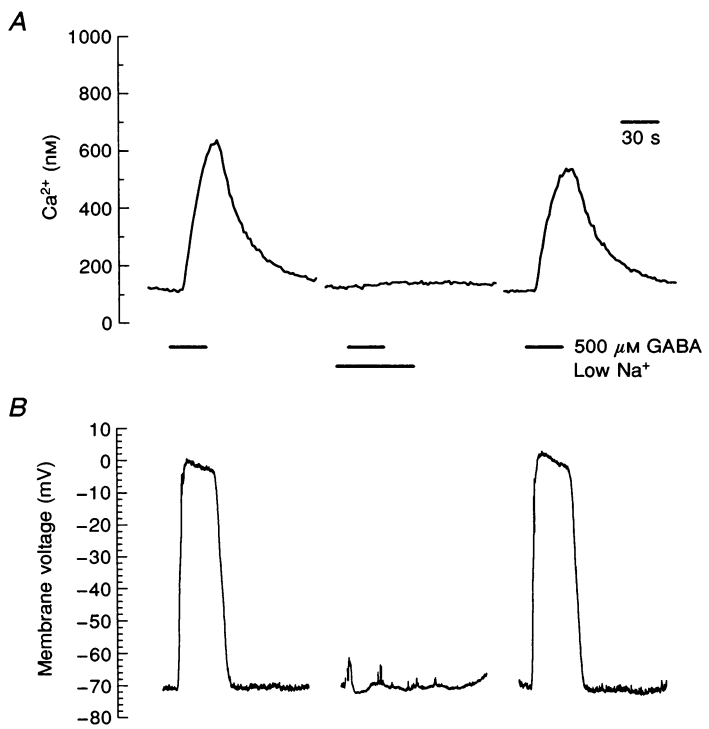

Figure 3. Effect of removing extracellular Na^+ on the GABA-induced rise in $[Ca^{2+}]_i$

A, calcium measurements in normal Ringer (left), low-Na^+ Ringer (middle), and upon the return to normal Ringer solution (right). The low-Na^+ solution completely abolished the GABA-induced $[Ca^{2+}]_i$ response. *B*, voltage measurements from the same cell recorded under current clamp (0 nA). The depolarization induced by GABA was also eliminated by the removal of Na^+. Both effects were reversible; the GABA-induced $[Ca^{2+}]_i$ increase and the membrane depolarization were restored when Na^+ was reintroduced into the extracellular solution.

Ca^{2+} channels. As shown in Fig. 5*A*, the GABA-induced increase in $[Ca^{2+}]_i$ was almost completely blocked in the presence of nifedipine. However, in the absence of voltage clamp, 500 μM GABA still evoked large depolarizing potentials despite the nifedipine block of the Ca^{2+} response. Data obtained in thirteen cells showed that nifedipine reduced the GABA-induced increase in Ca^{2+} by 89 $\pm$ 5% (S.D.).

Despite the efficacy of nifedipine, evidence that the increase in $[Ca^{2+}]_i$ did not arise solely from an influx of Ca^{2+} through voltage-activated calcium channels is apparent from the experiments shown in Figs 6 and 7. Incubation with the hydantoin derivative dantrolene (Sigma), an agent that has been shown to inhibit Ca^{2+}-induced Ca^{2+} release from internal stores in various cell types (Desmedt & Hainaut, 1977; Hua, Nohmi & Kuba, 1993), served to markedly reduce the response to GABA (Fig. 6*A*). Measurements on nine cells indicated that the Ca^{2+} response elicited by 500 μM GABA was reduced by 49 $\pm$ 12% in the presence of 50 μM dantrolene. Moreover, direct stimulation of Ca^{2+} release from internal stores could be suppressed completely by this concentration of dantrolene. Exposing horizontal cells to 5 mM caffeine produced a large transient increase in $[Ca^{2+}]_i$ that was reversibly blocked by dantrolene (Fig. 6*B*), but was not affected by 100 μM nifedipine (Fig. 6*C*).

It is important to ensure that the reaction to dantrolene resulted solely from its purported effect on intracellular Ca^{2+} storage sites, i.e. that the drug does not also exert a

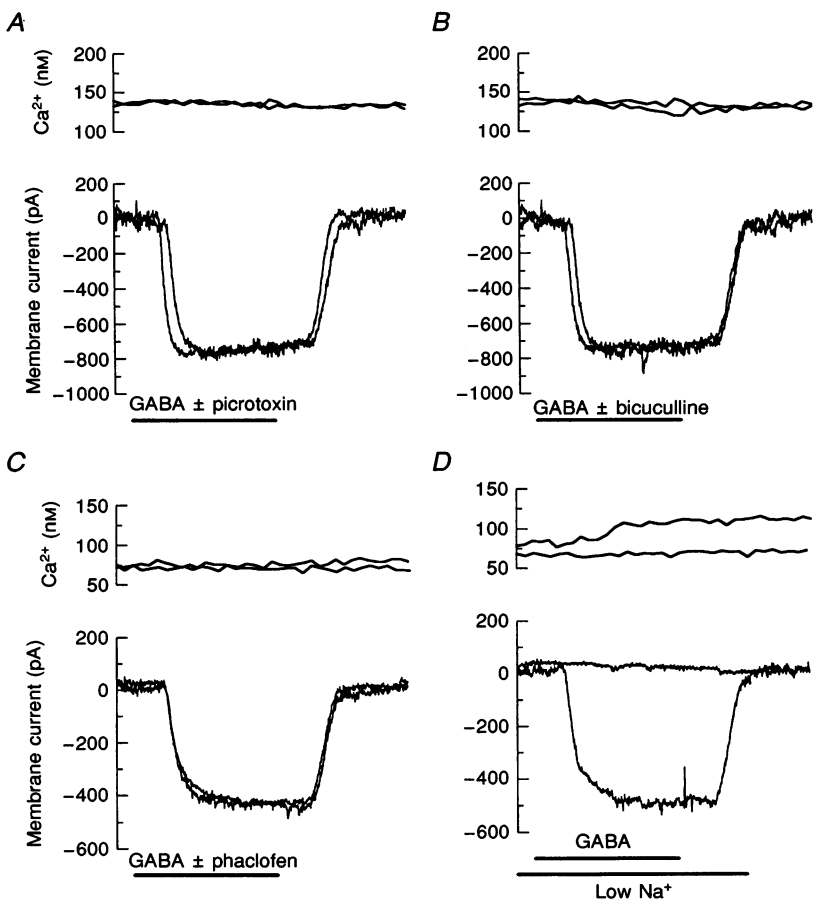

Figure 4. Membrane current and $[Ca^{2+}]_i$ changes in response to GABA

Recordings were obtained from voltage-clamped (−70 mV) horizontal cells in response to 30 s applications of 500 μM GABA with and without 250 μM picrotoxin (*A*), 250 μM bicuculline (*B*), 250 μM phaclofen (*C*), and a low-Na^+ solution (*D*). Each panel shows the response first to GABA alone, and then when GABA was co-applied with the test drug or in sodium-free Ringer solution. With the cells under voltage clamp, GABA activated an inward current, but did not elicit a change in $[Ca^{2+}]_i$. The GABA-induced current was not affected by the addition of picrotoxin, bicuculline or phaclofen, but the inward current was suppressed completely when Na^+ was replaced with 250 μM NMG^+ in the extracellular solution. Note also the slow increase in $[Ca^{2+}]_i$ in the low-sodium solution that was probably due to reversal of the Na^+–Ca^{2+} exchanger (see Discussion). The recordings are representative of results obtained on 6 cells.

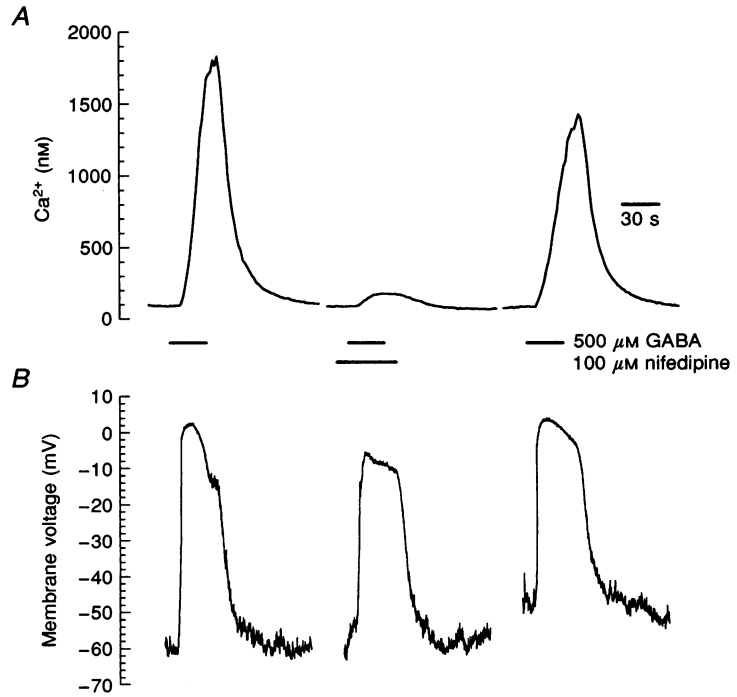

Figure 5. The effects of nifedipine on $[Ca^{2+}]_i$ and transmembrane potential

A, the GABA-induced increase in $[Ca^{2+}]_i$ was reversibly blocked by 100 μM nifedipine, an agent that blocks L-type voltage-gated Ca^{2+} channels. *B*, the simultaneously recorded voltage traces show that the membrane depolarization induced by GABA was not affected by nifedipine. Similar results were observed in 13 cells.

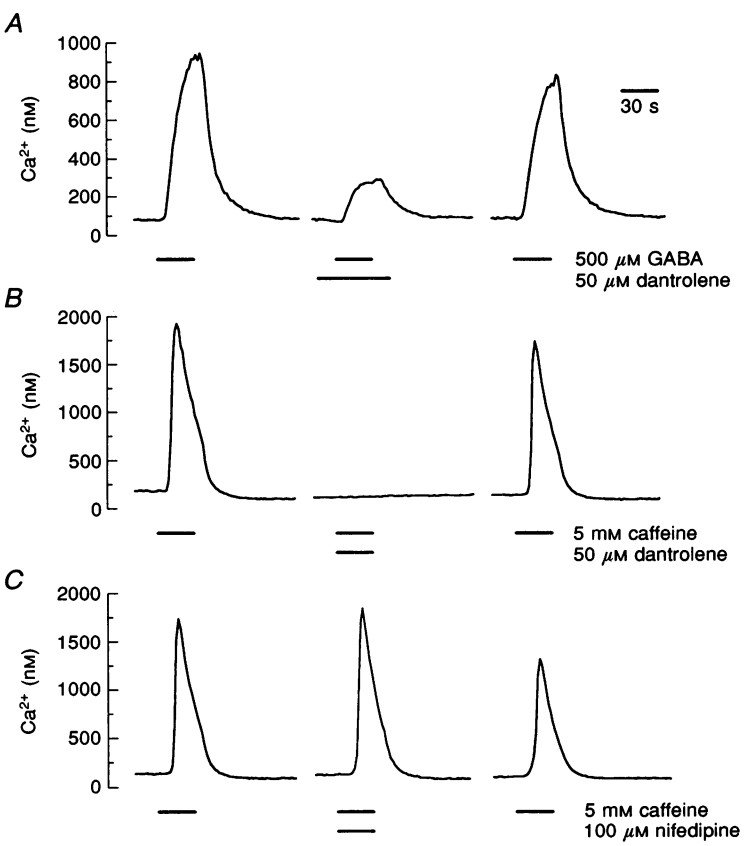

Figure 6. The GABA-induced rise in $[Ca^{2+}]_i$ derives in part from internal stores

A, the GABA-induced $[Ca^{2+}]_i$ increase was reduced by 50 μM dantrolene, a compound that blocks Ca^{2+} release from internal stores. *B*, 5 mM caffeine caused a rapid, transient rise in $[Ca^{2+}]_i$; the effect was suppressed completely by dantrolene. *C*, the caffeine-induced $[Ca^{2+}]_i$ increase was not affected by 100 μM nifedipine, i.e. the release from internal stores was not dependent upon Ca^{2+} entry. The cell in *A* was not under voltage clamp; cells in *B* and *C* were voltage clamped at −70 mV.

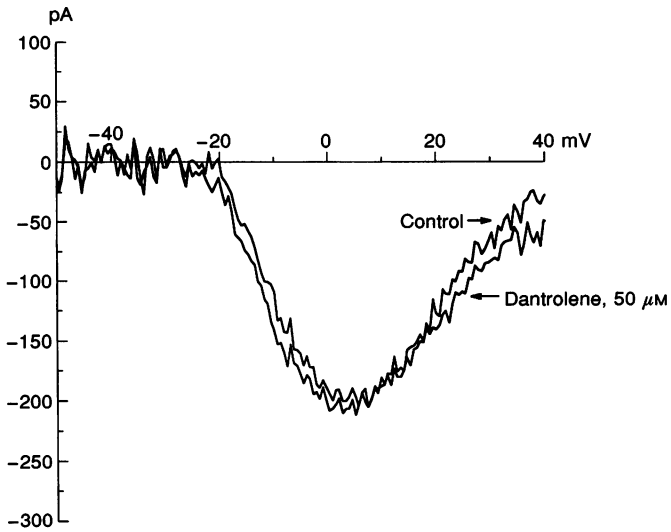

Figure 7. Effect of dantrolene on the voltage-activated Ca^{2+} conductance

Ca^{2+} was replaced with 4 mM Ba^{2+}, which more readily passes through voltage-gated Ca^{2+} channels. The horizontal cell was held at −70 mV and ramped to +40 mV at a rate of 150 mV s^{-1}. Currents were measured in control solution, in 50 μM dantrolene, and then in 100 μM nifedipine. The dihydropyridine-sensitive current was determined by subtracting the current measured in either control Ringer solution or dantrolene from that obtained in nifedipine. The nifedipine-blockable current had a peak at about 0 mV, and was not significantly affected by 50 μM dantrolene. The extracellular solution contained tetrodotoxin (0·5 μM), tetraethylammonium chloride (20 mM) and 4-aminopyridine (10 mM) to block voltage-gated Na^+ and K^+ channels. Similar results were obtained from 12 cells.

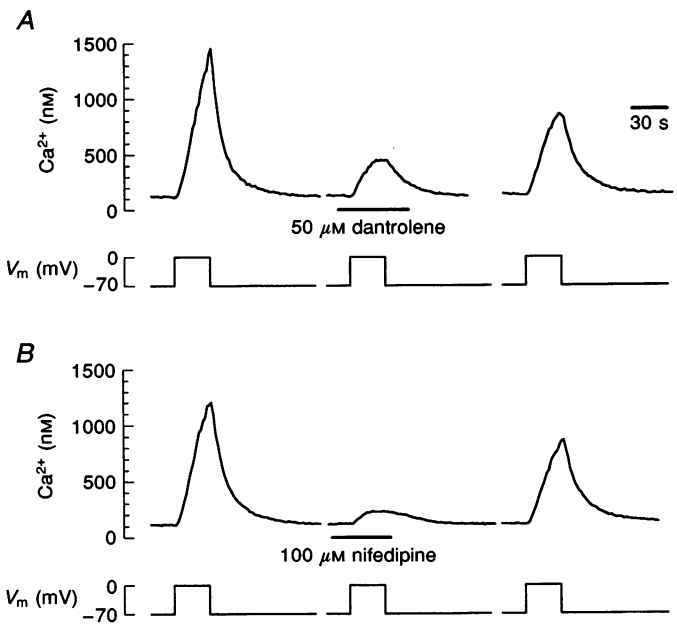

Figure 8. Intracellular Ca^{2+} responses to membrane depolarization

The horizontal cell (voltage clamped; V_m, membrane potential) was stepped from −70 to 0 mV and held at 0 mV for 30 s. *A*, in the presence of 50 μM dantrolene, the $[Ca^{2+}]_i$ increase was reduced. *B*, in the presence of 100 μM nifedipine, the $[Ca^{2+}]_i$ increase was almost completely blocked.

direct action on the voltage-gated calcium conductance. Figure 7 shows that the nifedipine-sensitive Ca^{2+} current, recorded under voltage clamp in response to a depolarizing voltage ramp, was not changed significantly by the addition of 50 μM dantrolene. Data from twelve cells indicated that the effect of dantrolene on the voltage-sensitive calcium current (near the peak of the response, i.e. in the range of -5 to 0 mV) was $0{\cdot}3 \pm 6{\cdot}2\%$ of the peak response in the control solution.

Assuming the GABA-mediated Ca^{2+} response is a consequence of the membrane depolarization induced by GABA uptake, it should be possible to mimic the effects of GABA by intracellular current injection. The series of recordings in Fig. 8 shows that this is indeed the case. Under whole-cell voltage clamp, stepping the membrane potential from -70 to 0 mV for 30 s raised the level of $[Ca^{2+}]_i$ by approximately 500 nM. In addition, the voltage-activated Ca^{2+} response was reduced $54 \pm 20\%$ ($n = 12$) by 50 μM dantrolene, and almost completely blocked by 100 μM nifedipine ($88 \pm 4\%$, $n = 14$).

DISCUSSION

The results of the present study demonstrate that GABA initiates a sequence of events that leads ultimately to a rise in intracellular calcium in isolated horizontal cells from the skate retina. The Ca^{2+} response has two principal components: (1) opening of voltage-activated Ca^{2+} channels that allow an influx of calcium, and (2) a subsequent calcium-induced calcium release from intracellular storage sites. Our findings suggest that the calcium increase is mediated by the sodium-dependent transport of GABA into the cells, an electrogenic process that promotes membrane depolarization. The depolarization activates L-type calcium channels which result in an influx of calcium from the extracellular solution; the latter triggers, in turn, the release of calcium from internal stores.

Ligand-gated GABA receptors

Although activation of $GABA_A$ and $GABA_B$ receptors is known to influence cytoplasmic levels of calcium (Heidelberger & Matthews, 1991; De Erausquin, Brooker, Costa & Wojcik, 1992; Reichling, Kyrozis, Wang & MacDermott, 1994), the pharmacological profile of the GABA-induced rise in $[Ca^{2+}]_i$ indicates that the response is not mediated by any of the known types of ligand-gated GABA receptor (Figs 2 and 4). Thus, the fact that bicuculline, picrotoxin and phaclofen had no effect on either the GABA-induced inward current (Fig. 4) or the concomitant rise in intracellular calcium (Fig. 2) would tend to rule out the possibility that GABA exerts its effect via activation of $GABA_A$ or $GABA_B$ receptors (cf. Malchow & Ripps, 1990). It is also unlikely that $GABA_C$ receptors are involved, since picrotoxin also blocks activation of the chloride channels associated with this conductance (Qian & Dowling, 1993).

The GABA-induced rise in $[Ca^{2+}]_i$ is mediated by GABA transport

There is compelling evidence indicating that the GABA-induced increase in $[Ca^{2+}]_i$ is mediated solely by a sodium-dependent GABA transport mechanism. In fact, removal of extracellular sodium abolished the GABA-induced inward current, the concomitant change in membrane voltage, and the increase in intracellular calcium (Figs 3 and 4). Moreover, the range of concentrations over which GABA elicited graded increases in $[Ca^{2+}]_i$, i.e. 50–500 μM, was consistent with the concentration dependence of the GABA-mediated transport current, where a half-maximal response was induced by 110 μM GABA (Malchow & Ripps, 1990).

Membrane depolarization links GABA transport with changes in $[Ca^{2+}]_i$

The GABA-induced rise in $[Ca^{2+}]_i$ was critically dependent upon membrane depolarization (Figs 1 and 8). At the resting potential of the isolated horizontal cell (approximately -75 mV), the small positive changes in membrane potential elicited by 10 or 20 μM GABA failed to produce a change in $[Ca^{2+}]_i$, nor was there an immediate Ca^{2+} response to 50 or 100 μM GABA, which initially depolarized the cell to about -50 mV. At this voltage level, however, the opening of sodium channels and activation of a calcium conductance probably come into play to trigger a spike-like depolarizing response and a large influx of Ca^{2+} (Malchow *et al.* 1990; Sullivan & Lasater, 1992). Once the threshold was exceeded, the onset, magnitude and duration of the increase in $[Ca^{2+}]_i$ was governed in part by the latency and duration of the depolarizing potential.

It is noteworthy that application of 100 μM GABA produced depolarizing potentials that did not overshoot 0 mV, nor was there evidence for rapid desensitization; similar phenomena were reported by Lasater, Dowling & Ripps (1984). It would appear that for these typically non-spiking cells, the seemingly 'all-or-none' response reflects the net result of the multiple voltage-gated conductances of skate horizontal cells (Malchow *et al.* 1990). As we have shown previously, depolarization evokes an inward calcium current of the order of 0·02 pA μm^2 in addition to a TEA-blockable outward current of approximately the same amplitude. There is also a TTX-blockable sodium current in these cells, which probably contributes to the rapid initial rise in voltage; the expression of this current is highly transient and not likely to be detected at the sampling rate used in these experiments. With regard to the sustained depolarization, this is probably due to the balance between the above mentioned currents and the current elicited by the electrogenic transport of GABA.

The GABA-induced rise in $[Ca^{2+}]_i$ derives in part from Ca^{2+}-induced Ca^{2+} release

Recent studies have shown that teleost horizontal cells have the capacity to sequester large amounts of calcium, and that Ca^{2+} release from intracellular storage sites can be effected by exposure to caffeine (cf. Yasui, 1988; Linn & Christensen, 1992). This appears to be the case also in the elasmobranch, where a significant component of the rise in intracellular calcium clearly derives from intracellular stores. The release of large amounts of Ca^{2+} from these stores is evident in Fig. 6*B* and *C*; caffeine (5 mM) produced increases in $[Ca^{2+}]_i$ at least as great as that resulting from 500 μM GABA. Moreover, the fact that 50 μM dantrolene blocked completely the caffeine response (Fig. 6*B*), but had no significant effect on the voltage-activated Ca^{2+} channel (Fig. 7), makes it possible to estimate the magnitude of the contribution from internal stores to the GABA-induced calcium response. Thus, after eliminating Ca^{2+} release from internal stores with dantrolene, the observation that the GABA-induced rise in $[Ca^{2+}]_i$ was reduced by about 50% (Fig. 6*A*) suggests that approximately 50% of the rise in internal calcium is derived from calcium-induced calcium release. Obviously, the entry of extracellular calcium through the voltage-sensitive calcium channels not only accounts for the other half of the calcium increase, but is required to activate the Ca^{2+} release from internal stores. This is evident in the data of Fig. 5; blocking the voltage-dependent Ca^{2+} conductance with nifedipine had no effect on membrane depolarization, but reduced the rise in $[Ca^{2+}]_i$ by more than 90%.

Na^+–Ca^{2+} exchange may be affected by GABA transport

A small calcium rise remained when Ca^{2+} influx through voltage-gated channels was inhibited by nifedipine (Fig. 5). The calcium increase could result from an incomplete block of calcium channels at this concentration of nifedipine. Alternatively, the rise in calcium may reflect reversal of a Na^+–Ca^{2+} exchanger that normally operates to extrude Ca^{2+} under physiological conditions (Yau & Nakatani, 1984; Yasui, 1988). Two Na^+ and one Cl^- accompany each molecule of GABA transported into the cell (Keynan & Kanner, 1988), resulting in an increased intracellular sodium load and membrane depolarization. These conditions probably induce a reversal of Na^+–Ca^{2+} exchange, i.e. the extrusion of Na^+ and the influx of Ca^{2+}, which may be greatly enhanced as a result of the depolarization-induced activation of an inward sodium current. A similar phenomenon has been purported to occur in cardiac myocytes (Leblanc & Hume, 1990; Levi, Brooksby & Hancox, 1993). In an analogous manner, altering the Na^+ gradient by removing extracellular Na^+ would reverse the directionality of the Na^+–Ca^{2+} exchanger (Carvalho, Bandeira-Duarte, Ferreira, Coutinho & Carvalho, 1991), and account for the slow influx of Ca^{2+} observed in Fig. 3.

$[Ca^{2+}]_i$ and modulation of neuronal activity

Although there is good reason to suppose that the GABA transporter of skate horizontal cells plays a key role in the removal of GABA and terminating its synaptic activity in the distal retina, the physiological significance of the GABA-induced depolarization and associated rise in $[Ca^{2+}]_i$ is not immediately apparent. Both effects may serve to stimulate GABA release. In the vertebrate retina, the discharge of GABA from horizontal cells appears to occur via Ca^{2+}-dependent vesicular release (Marshak & Dowling, 1984; Sakai & Naka, 1986) and a Ca^{2+}-independent mechanism mediated by reversal of the GABA transporter (Yazulla & Kleinschmidt, 1983; Schwartz, 1987). The depolarization induced by GABA uptake could serve to promote its own discharge by stimulating vesicular release due to increased internal calcium levels. It is also possible that reversal of the GABA transporter due to its voltage dependence and the increased $[Na^+]_i$ resulting from activation of voltage-dependent sodium channels might promote the release of GABA (Schwartz, 1987; Cammack & Schwartz, 1993). In either event, the release of GABA from the horizontal cells could increase synaptic inhibition within the distal retina. The question of whether the GABA concentration at synaptic sites within the intact retina is sufficiently high to induce these effects has yet to be addressed adequately. Indeed, it is likely that horizontal cells *in vivo* have a significantly lower input resistance than isolated cells owing to the maintained discharge of glutamate from photoreceptor terminals; this would tend to reduce the depolarizing response to GABA.

Another potentially important consequence of GABA uptake relates to the subsequent rise in $[Ca^{2+}]_i$, and the modulation of gap-junctional communication. In all vertebrate species studied thus far, horizontal cells are coupled electrically through an extensive series of intercellular gap junctions (cf. Naka & Rushton, 1967; Lamb, 1976; Qian & Ripps, 1992). Calcium has been shown to be an effective modulator of the ionic conductances through the pores formed by the proteins (connexins) that constitute the gap junction. Through molecular events that have yet to be completely identified, a rise in intracellular calcium reduces intercellular coupling, thereby restricting the spread of current throughout the network of coupled cells (Rose & Loewenstein, 1976; Crow, Atkinson & Johnson, 1994). Thus, fluctuations in intracellular calcium may affect the receptive-field organization of the horizontal cells, the transfer of signals across the retina, and the influence exerted by the horizontal cells on the sensitivity of other neurons in the visual pathway. To what extent such changes occur under the physiological conditions encountered *in vivo* is not known; studies on the slice preparation and intact retina will be needed to address these issues more definitively.

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