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# CALCIUM CHANNELS IN SOLITARY RETINAL GANGLION CELLS FROM POST-NATAL RAT

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## SUMMARY

1. Calcium currents from identified, post-natal retinal ganglion cell neurones from rat were studied with whole-cell and single-channel patch-clamp techniques. $Na^+$ and $K^+$ currents were suppressed with pharmacological agents, allowing isolation of current carried by either 10 mM-$Ca^{2+}$ or $Ba^{2+}$ during whole-cell recordings. For cell-attached patch recordings, the recording pipette contained 96–110 mM-$BaCl_2$ while the bath solution consisted of isotonic potassium aspartate in order to zero the neuronal membrane potential.

2. A transient component, present in approximately one-third of the whole-cell recordings resembles closely the T-type calcium current observed previously in other tissues. This component activates at low voltages (−40 to −50 mV from holding potentials negative to −80 mV), inactivates with a time constant of 10–30 ms at 35 °C, and is carried equally well by $Ba^{2+}$ or $Ca^{2+}$. In single-channel recordings small (8 pS) channels are observed whose aggregate microscopic kinetics correspond well to the macroscopic current obtained during whole-cell measurements.

3. During whole-cell recordings, a more prolonged component activates in all retinal ganglion cells at −40 to −20 mV from a holding potential of −90 mV. This component is substantially larger when equimolar $Ba^{2+}$ replaces $Ca^{2+}$ as the charge carrier, and is sensitive to the dihydropyridine agonist Bay K8644 (5 $\mu$M) and antagonists nifedipine (1–10 $\mu$M) and nimodipine (1–10 $\mu$M). Thus, the dihydropyridine pharmacology of this prolonged component resembles that of the L-type calcium current found in dorsal root ganglion neurones and in heart cells. Also reminiscent of the L-current, the prolonged component in this preparation is less inactivated at depolarized holding potentials (−60 to −40 mV) than the transient component. In cell-attached recordings, large (20 pS) channels are observed with activation properties similar to those of the prolonged portion of the whole-cell current.

4. $\omega$-Conotoxin fraction GVIA ($\omega$-CgTX VIA), a peptide from the venom of the snail *Conus geographus*, produces a readily reversible blockade of all components of the calcium current in these central mammalian neurones. This finding is in contrast

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to that of other preparations in which this toxin is responsible for an ephemeral block of T-current but a long-lasting block of other components of calcium current.

5. In summary, at least two components of calcium current with discrete underlying unitary events are present in post-natal retinal ganglion cells from rat. One component closely resembles the T or transient current observed in other cell types. The second type displays a prolonged component and several pharmacological properties similar to those of the previously described L-type calcium current; nevertheless, the prolonged component is clearly different from L-current since in this preparation it is blocked by ω-CgTX VIA in a rapidly reversible manner.

## INTRODUCTION

Calcium ion fluxes through voltage-dependent calcium channels have been implicated in cellular functions as diverse as neurite outgrowth, gene regulation, neurotransmitter release, and muscle contraction. Multiple types of calcium channel have been described in different types of tissue (for a review, see Bean, 1989). For example, two types of calcium channel have been described in voltage-clamp studies in some preparations (for example, Hagiwara, Ozawa & Sand, 1975; Fishman & Spector, 1981; Carbone & Lux, 1984*a*, *b*; Deitmer, 1984; Fox & Krasne, 1984; Cota & Stefani, 1985; Nilius, Hess, Lansman & Tsien, 1985; Matteson & Armstrong, 1986; Carbone & Lux, 1987; Narahashi, Tsunoo & Yoshii, 1987; Yaari, Hamon & Lux, 1987). Furthermore, three components of calcium current have been observed in chick dorsal root ganglion (DRG) neurones (Nowycky, Fox & Tsien, 1985; Fox, Nowycky & Tsien, 1987*a*, *b*) and in embryonic mouse DRG neurones (Kostyuk, Shuba & Savchenko, 1988). However, by analysing tail currents on a microsecond time scale Swandulla & Armstrong (1988) have disputed the existence of the third or N-type calcium current in these neurones. They found evidence for only two components, a transient (T-type) or low-voltage activated current, and a more prolonged (L-type) or high-voltage activated current.

Recently it has been claimed that particular subtypes of calcium channel may subserve specific cellular activities. For example, in rat sympathetic neurones two types of calcium channel have been described, an L-type, which contributes maintained currents from depolarized holding potentials and an N-type, which slowly inactivates during maintained depolarizations and requires hyperpolarized holding potentials to reactivate; the N-type channels have been implicated in neurotransmitter release (Hirning, Fox, McCleskey, Olivera, Thayer, Miller & Tsien, 1988). Central to these kinds of experiment is the ability to separate pharmacologically the various types of calcium channel. A peptide from the venom of the snail *Conus geographus*, ω-conotoxin GVIA (ω-CgTX VIA), produces a persistent blockade of the N and L but not the T components of calcium current in chick DRG neurones (McClesky, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987). Therefore, this toxin has been used extensively to separate these components of calcium current. In this paper we present evidence for at least two types of calcium current in a post-natal mammalian central neurone, the rat retinal ganglion cell. Although these calcium currents share several properties with those previously described for other tissues, for example one is very similar to the T-type and the other somewhat

reminiscent of the L-type, they are also clearly different. Although in retinal ganglion cells ω-CgTX exerts a powerful antagonistic effect on both components of the calcium current, this inhibition is rapidly reversible and not persistent. Thus, the toxin cannot be used to distinguish between the types of calcium channel in this central neuronal preparation. These studies suggest that the pharmacology of a specific component of calcium current may be peculiar to the cell type under study.

## METHODS

### *Identification, dissociation and tissue culture of neurones*

Rat retinal ganglion cells were labelled with the fluorescent marker Granular Blue following retrograde transport of the dye from the superior colliculus *in situ*. The dissociation and culture techniques have been described previously in detail (Leifer, Lipton, Barnstable & Masland, 1984; Lipton & Tauck, 1987). In general, retinal cells were obtained from 7- to 12-day-old Long–Evans rat pups. All procedures were performed under anaesthesia (cryoanaesthesia for young, hairless animals and fluorane inhalant anaesthesia for older animals). Briefly, following cervical dislocation and enucleation, the retina was gently dissociated by trituration through a glass pipette following digestion with papain. The isolated ganglion cells could be unequivocally identified by the presence of fluorescent dye (for colour photographs, see Leifer *et al.* 1984; Lipton, Wagner, Madison & D'Amore, 1988). The retinal cells were cultured in Eagle's minimum essential medium; fetal calf serum or rat serum (5% v/v); glutamine (2 mM); methylcellulose (0·7% w/v); glucose (16 mM); and gentamicin (1 μg/ml). Ganglion cells were generally plated on plain glass or polylysine-coated glass cover-slips for electrophysiological recordings; under these conditions the neurones displayed a spatially compact configuration, lacking processes (Leifer *et al.* 1984). A subpopulation of retinal ganglion cell neurones could be identified based upon their large size and staining characteristics with antibodies against the 150 kDa subunit of neurofilaments (Dräger & Hofbauer, 1984; S. A. Lipton & U. C. Dräger, unpublished observations). These ganglion cells with large somas corresponded to the α- or Y-like cells in the intact retina (Fukada, 1977; Perry, 1979). Although we could distinguish at least two subclasses of mammalian retinal ganglion cells (with large and small somas), our findings pertaining to calcium currents were not different with respect to a particular subtype

### *Recording conditions and superfusion system*

Whole-cell and cell-attached patch recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) were performed as described previously for mammalian retinal ganglion cell neurones (Lipton & Tauck, 1987). Retinal ganglion cells were used 4–20 h after plating without obvious differences in the results. Experiments were generally performed at 33–36 °C. For whole-cell recordings, cells without processes were chosen to ensure an adequate space clamp. Gigaohm seals were generally obtained on retinal ganglion cells bathed in modified Hanks' balanced salt solution, composition (in mM): NaCl, 137·6; KCl, 5·8; $CaCl_2$, 2·5; $MgCl_2$, 1; HEPES–NaOH, 5; glucose, 22·2; Phenol Red indicator (0·001% v/v), pH 7·2. To prevent the precipitation of calcium and barium salts at the tips of the patch pipettes, sulphate and phosphate were eliminated in this physiological saline. The cells were continually superfused at 1–2 ml/min in a chamber with a volume of 100 μl. After forming a seal for whole-cell recording (and occasionally before sealing the patch pipette to the cell surface), the bathing fluid superfusing the cell was changed to a solution that in conjunction with the patch pipette solution would minimize interference of $Na^+$ and $K^+$ currents; another technique used was the application of a second bathing medium by a pneumatic pipette placed within 20 μm of the neurone. In some experiments this medium consisted of (in mM): tetraethylammonium chloride (TEA-Cl) or choline chloride, 120; $BaCl_2$ or $CaCl_2$, 10; $MgCl_2$, 2; HEPES, 10; pH 7·2 with TEA-OH. Since retinal ganglion cells often deteriorated within minutes after being exposed to this bath solution (Lipton & Tauck, 1987), other experiments used the modified Hanks' balanced salt medium listed above with 10 mM-$CaCl_2$ or 10 mM-$BaCl_2$ instead of 2·5 mM-$CaCl_2$ and with the addition of tetrodotoxin (TTX, 1 μM). This substitution yielded the same results as the tetraethylammonium or choline chloride solutions but the recordings were stable for much longer periods of time. Agonist and antagonist dihydropyridines (Bay K 8644, nifedipine and nimodipine) were stored in the dark and used under conditions of low illumination.

For whole-cell recordings, $K^+$ currents (Lipton & Tauck, 1987) were suppressed by using a caesium- and TEA-containing solution in the patch pipette (composition in mM): CsCl or caesium aspartate, 120; TEA-Cl, 5–20; EGTA or BAPTA (bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid), 1·5–11; $CaCl_2$, 0–1; $MgCl_2$, 1; HEPES, 10; ATP, 3–3·6, sometimes with 50 U/ml creatine phosphokinase and 14·2 mM-phosphocreatine to decrease $Ca^{2+}$ current run-down; pH 7·2 with TEA-OH; occasionally this solution also contained 1 mM-GTP. With 10 mM-EGTA, 1 mM-$MgCl_2$, and no added $Ca^{2+}$ in the pipette, the calculated free $Ca^{2+}$ was nearly as low as $10^{-9}$ M. When the pipette contained 11 mM-EGTA, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$, the calculated free $Ca^{2+}$ was $2 \times 10^{-8}$ M. With 1·5 mM-EGTA, 1 mM-$MgCl_2$ and 1 mM-$CaCl_2$, the free $Ca^{2+}$ was $4 \times 10^{-7}$ M (Caldwell, 1970). The latter solution, with the highest level of free $Ca^{2+}$ being dialysed into the cell, sometimes appeared to hasten the run-down of calcium current; otherwise results obtained with all three levels of free $Ca^{2+}$ were qualitatively similar. The exact composition for the bath and patch pipette solutions used in individual recordings is listed in each figure legend.

For single-channel recordings, performed in the cell-attached configuration, the pipette contained 96–110 mM-$BaCl_2$, 10 mM-HEPES, and occasionally 1 μM-TTX (tetrodotoxin) and 100 μM-3,4-diaminopyridine (pH 7·2 with TEA-OH). The TEA, 3,4-diaminopyridine and TTX were added to ensure that $Na^+$ and $K^+$ channels were blocked. Recordings obtained with and without these drugs, however, were similar. During the cell-attached recordings, the bath solution was exchanged for (in mM): potassium aspartate, 140; K-EGTA, 10; $MgCl_2$, 1; HEPES, 10 (pH 7·2 with KOH) to zero the cell resting potential (Nowycky *et al.* 1985; Fox *et al.* 1987*b*). This procedure was verified by measuring the reversal potential of non-selective cation currents in the patch while cell attached and following excision (Lipton, 1987).

### *Data acquisition and analysis*

Current signals were sampled on-line after being elicited by test potentials generated by a PDP 11/23 or 11/73 computer (Digital Equipment Corp., Maynard, MA, USA) with a Cheshire Data interface (Hamden, CT, USA). The evoked currents were measured with an EPC-7 patch-clamp amplifier (List Electronic, FRG). Whole cell currents were generally sampled at a rate of 5–10 kHz with a 12-bit analog-to-digital converter (Data Translation, Marlboro, MA, USA) and filtered with a 48 dB/octave Bessel characteristic at 1–5 kHz. Corrections for series resistance, leakage currents, and liquid junction potentials were made as previously reported (Lipton & Tauck, 1987). The capacitance of the pipette was offset during cell-attached recording using the internal compensation circuitry of the patch-clamp amplifier. For single-channel recording in the cell-attached mode, currents were sampled at 5 kHz and filtered at 1 kHz. The average of several traces lacking channel openings was used to subtract digitally the linear leak and capacity transients. For some patches in which null traces were too infrequent to yield an adequate average, the capacity transients were clipped and the leak subtracted by the technique of Fox *et al.* (1987*b*) in which a few null traces were fitted by eye with the sum of two exponentials to form a smooth trace for leak subtraction. Total amplitude histograms of single-channel recordings were obtained by using sample points in traces that had been leak subtracted but not idealized. Gaussians were fitted by eye to the data in the histograms of current level (such as that illustrated in Fig. 6*C*). Analysis of both whole-cell and single-channel records was performed off-line using programs provided by Drs M. P. Frosch, P. Hess, and M. Plummer. Calcium currents were analysed in a total of 175 retinal ganglion cells for this study (147 whole-cell recordings and 28 cell-attached patch recordings).

## RESULTS

### *Types of $Ca^{2+}$ current in whole-cell recordings of retinal ganglion cells*

### *Predominance of separate $Ca^{2+}$ current components in different cells*

The whole-cell configuration of the patch-clamp technique was used to record $Ca^{2+}$ currents from identified, post-natal rat retinal ganglion cells. A combination of solutions in the bath and pipette (see Methods) was utilized to ensure complete isolation of $Ca^{2+}$ current from the other currents that have been described in these mammalian central neurones (Lipton & Tauck, 1987). For example, $Na^+$ current was

blocked using TTX and occasionally with the complete replacement of external $Na^+$ in the bathing solution with either choline or TEA. Under these conditions, robust currents through $Ca^{2+}$ channels were recorded using either 10 mM-$Ca^{2+}$ or 10 mM-$Ba^{2+}$ as the charge carrier. Previously, it had been shown that in this preparation the calcium current isolated in this manner could be completely blocked by $Co^{2+}$ or $Cd^{2+}$ (Lipton & Tauck, 1987).

The amplitudes of the $Ca^{2+}$ currents irreversibly decreased after establishing a whole-cell recording and finally disappeared after 10–15 min. This 'run-down' of $Ca^{2+}$ currents, as described in other dialysed cell types (Kostyuk, Veselovsky & Fedulova, 1981), was accelerated by repetitive and long-lasting depolarizing test pulses which were therefore avoided. To reduce this run-down, which is probably due to wash-out of an intracellular component during whole-cell recording, ATP and often creatine phosphokinase and phosphocreatine were added to the patch pipette solution and were effective (Byerly & Yazejian, 1986). Figure 1 shows $Ca^{2+}$ currents elicited by depolarizing voltage steps applied from a holding potential of −90 mV. In these recordings obtained in three different cells, and in many similar experiments, a small transient component could often be seen at −50 to −30 mV (present in $n = 50/147$ or 34% of the whole-cell recordings). The inactivation of the transient current could be fitted with a single exponential with a time constant of 10–30 ms. In addition, a sustained current became evident at −40 to −20 mV in every retinal ganglion cell. Longer steps revealed that the duration of the sustained current was in excess of 2 s. Finally, at or above −20 mV a more slowly decaying exponential parameter was frequently needed to fit the current ($\tau$ = 100–200 ms; observed in $n = 118/147$ cells or 80%). The mere presence of three different kinetically fitted parameters to the current does not, however, necessarily indicate that three discrete types of $Ca^{2+}$ channel underlie these responses. Nevertheless, the pharmacological and single-channel evidence presented below does allow separation of at least two types of calcium channel in this preparation. A transient current component is, to some degree, analogous to the T-type observed in many tissues including DRG neurones by Fox *et al.* (1987*a*): therefore, this nomenclature will be used here with the caveat that further studies may well define differences between this type of calcium current in chick DRG neurones and in rat retinal ganglion cell neurones. A more prolonged calcium current in mammalian retinal ganglion cells is pharmacologically distinct from the L-type current found in chick DRG neurones or in heart muscle (*vide infra*), so the term 'L-type' will not be used here in order to avoid confusion.

Figure 1*A* shows an example of a retinal ganglion cell with robust sustained and transient components but relatively little slow decay. In contrast, Fig. 1*B* displays currents from a different retinal ganglion cell with large transient and prolonged components, the latter being fitted by a slowly decaying kinetic parameter and with a sustained phase. Compare the currents elicited by voltage steps to −20 or to −10 mV in Fig. 1*A* and *B*. It can be clearly seen in these traces that the current fitted with a slowly decaying parameter was larger and appeared to activate earlier in the latter example. Still another variation can be observed in Fig. 1*C* in which only a very minor, if any, contribution from the T component is found in another retinal ganglion cell. From the evidence to be presented in this study, the slowly decaying phase may be due to the voltage- or current-dependent decay of the sustained

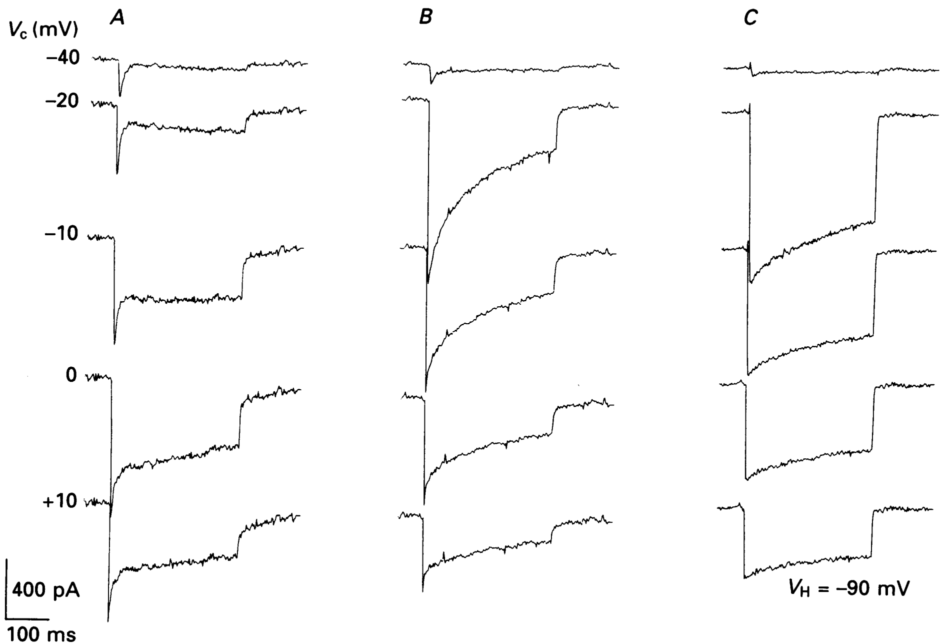


Fig. 1. Variation in the transient and prolonged components of calcium current in whole-cell recordings from disparate rat retinal ganglion cells. Whole-cell calcium currents with either $Ba^{2+}$ or $Ca^{2+}$ as the charge carrier were obtained from rat retinal ganglion cells in response to a series of voltage-clamp steps from a holding potential ($V_H$) of −90 mV. The magnitude of the command potential ($V_C$) is listed adjacent to the current that it evoked. *A*, a cell with large transient and sustained phases of calcium current but a relatively small contribution of a slowly decaying phase. *B*, another ganglion cell with large transient, slowly decaying, and sustained phases. *C*, a third cell with a robust prolonged component (comprising sustained and slowly decaying phases) but little transient component. The bath solution consisted of a modification of Hanks' balanced salts (in mM): NaCl, 137·6; KCl, 5·8; $MgCl_2$, 1; HEPES–NaOH, 5; glucose, 22·2; Phenol Red indicator (0·001 % v/v), pH 7·2; plus 1 μM-TTX and 10 mM-$CaCl_2$ in *A* and *B* or 10 mM-$BaCl_2$ in *C*. The patch pipette solution contained (in mM): CsCl, 120; TEA-Cl, 20; HEPES, 10; EGTA, 1·5; $CaCl_2$, 1; $MgCl_2$, 1; (plus ATP, 3; GTP, 1 in *C*); pH 7·2. Temperature 35 °C.

current since no pharmacological or voltage-dependent separation of these entities is possible in whole-cell recordings and since, to date, single-channel recordings have yielded definitive evidence for only two types of unitary events in our preparation (*vide infra*; cf. Hagiwara, Irisawa & Kameyama, 1988). Thus, the term 'prolonged' component of calcium current will be used herein to describe this second type and to distinguish it from the transient component.

It is less likely that the slowly decaying phase of the prolonged component of calcium current is due to current-dependent inactivation (rather than to voltage-dependent inactivation) of the sustained phase of the current for two reasons: (1) slow decay was still observed when the internal concentration of EGTA was raised from 1·5 to 11 mM in the presence of 1 mM-$CaCl_2$, or with 10 mM-EGTA (or 5 mM-BAPTA) in the absence of added $CaCl_2$, lowering the calculated free $Ca^{2+}$ from $4 \times 10^{-7}$ to nearly $10^{-9}$ M (see Methods); and (2) slowly decaying currents were also observed upon substitution of external $Ca^{2+}$ with $Ba^{2+}$ as the charge carrier; in general, $Ba^{2+}$ does not produce current-dependent inactivation like $Ca^{2+}$ (Eckert & Chad, 1984; see also Fox *et al.* 1987*b*).

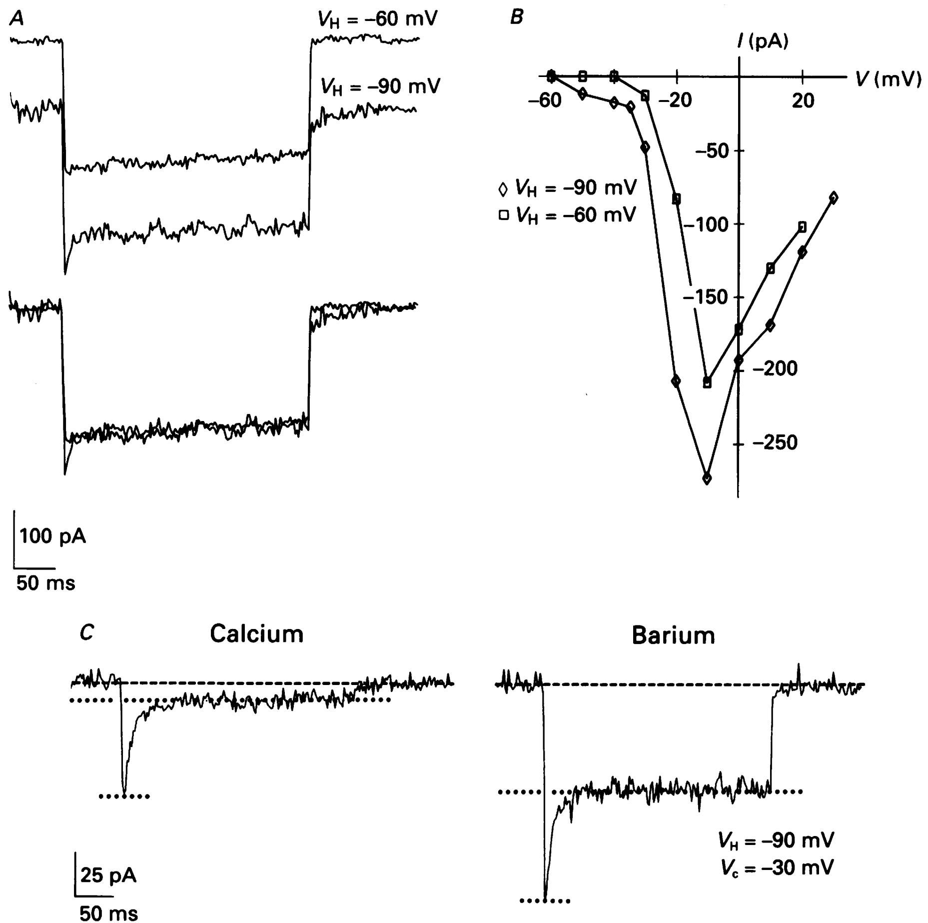


Fig. 2. Identification of two components of calcium currents in retinal ganglion cells by (*A* and *B*) depolarizing pulses from two different holding potentials, or (*C*) substitution of $Ca^{2+}$ for $Ba^{2+}$ as the charge carrier. *A*, separation of two calcium currents by varying the holding potential in whole-cell recordings. Depolarization from $V_H = -60$ instead of $-90$ mV eliminated the transient component of the calcium current as shown in the upper two representative traces evoked by $V_C = -10$ mV. In the lowermost set of traces these two responses are superimposed to illustrate that the prolonged component of the calcium current was essentially unaffected. *B*, *I–V* relationships for the same neurone at the two holding potentials. *C*, in a different retinal ganglion cell, transient current was nearly equal in magnitude when 10 mM-$Ca^{2+}$ was replaced with equimolar $Ba^{2+}$ as the charge carrier while the prolonged current was greatly enhanced. The transient component is indicated as current between the dotted lines. The bath solution was the same as in Fig. 1 with 10 mM-$Ba^{2+}$ as the charge carrier in *A* and *B* and 10 mM-$Ba^{2+}$ or 10 mM-$Ca^{2+}$, as indicated, in *C*. The patch pipette solution in *A* and *B* contained (in mM): CsCl, 120; TEA-Cl, 20; HEPES, 10; EGTA, 11; ATP, 3; GTP, 1; $CaCl_2$, 1; $MgCl_2$, 1; pH 7·2. In *C*, 5 mM-BAPTA was substituted for EGTA and no $CaCl_2$ or GTP was added to the solution.

These retinal ganglion cells were all nearly spherical in shape and lacked processes, which yielded an excellent space-clamped preparation. Therefore, the varying predominance of the various components of calcium current cannot be explained on the basis of poor recording techniques or configurations. However, because the cells

did not have neurites, only calcium channels in the cell body were studied. In principle, these channels are quite possibly different from those on the processes of intact neurones.

*Separation of two $Ca^{2+}$ current components by different holding potentials*

Figure 2*A* and *B* shows that the rapid, transient (T-type) component of the $Ca^{2+}$ current was inactivated by holding the membrane voltage depolarized at −60 rather than at −90 mV, while the more prolonged component was preserved. This finding is similar to that previously reported in DRG neurones (Nowycky *et al.* 1985; Fox *et al.* 1987*a*) as well as in other cell types. In Fig. 2*A* there is evidence for selective inactivation of one kinetic component, and the current–voltage (*I–V*) curves for the two holding potentials in Fig. 2*B* display a slightly different shape, at least over the range from −60 to −35 mV. Both of these points support the hypothesis that the transient and prolonged components of the calcium current are functionally distinguishable.

*T current remains relatively unchanged in high external $Ba^{2+}$ while the prolonged component is enhanced*

In embryonic chick DRG neurones (Fox *et al.* 1987*b*) and in fetal rat hippocampal nerve cells (Carbone & Lux, 1987), as well as in other cell types, substituting $Ba^{2+}$ for $Ca^{2+}$ leaves the amplitude of the T component approximately constant while increasing the other component(s). This finding appeared to hold in post-natal mammalian retinal ganglion cells as well. For example, in Fig. 2*C* the transient (T) component was relatively unaffected (1·1-fold increase) by sequentially changing external $Ca^{2+}$ to $Ba^{2+}$. In contrast, the prolonged component increased substantially (6-fold enhancement). Moreover, at a command potential of −30 mV the transient current accounted for 85% of the peak current in 10 mM-external $Ca^{2+}$ but only ~50% in equimolar $Ba^{2+}$. This differential influence of $Ba^{2+}$ and $Ca^{2+}$ on the amplitude of these components argues that the transient and prolonged currents have truly separate properties with respect to the charge carried through their channels. The substitution of $Ba^{2+}$ for $Ca^{2+}$ also resulted in a 5–10 mV shift to the left of the *I–V* curves for both the peak transient and prolonged currents.

*Pharmacology of dihydropyridines on calcium current*

The antagonist dihydropyridines, nifedipine and nimodipine, have been previously shown in other systems to preferentially block the L (sustained) component of $Ca^{2+}$ current, especially when the membrane is depolarized (Brown, Kunze & Yatani, 1986), stemming from the fact that dihydropyridine drug binding is promoted by either L-channel activation or inactivation (Bean, 1984; Sanguinetti & Kass, 1984). In retinal ganglion cells 1–10 μM-nifedipine or -nimodipine substantially inhibited the whole-cell $Ca^{2+}$ current with the exception of the transient component. As shown in Fig. 3, blockade was evident even at hyperpolarized holding potentials and with GTP in the pipette solution, both of which have been reported to decrease the effect of the antagonist dihydropyridines in rat DRG neurones (Scott & Dolphin, 1987, 1988).

In addition, in several tissues outside of the retina the agonist dihydropyridine, Bay K 8644, promotes prolonged openings of single L-type $Ca^{2+}$ channels and, thus,

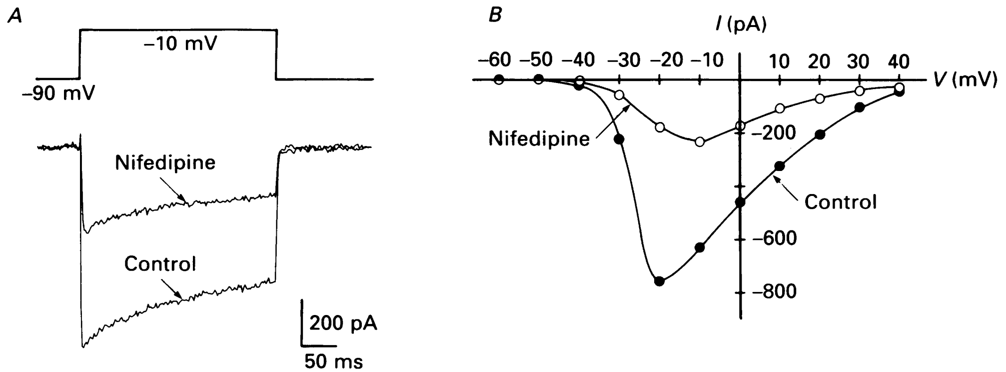


Fig. 3. Antagonist dihydropyridine, nifedipine, blocks whole-cell calcium current in retinal ganglion cells. *A*, with $Ba^{2+}$ as the charge carrier, nifedipine (10 $\mu$M) inhibited 60% of the current elicited by a voltage step from $V_H = -90$ mV to $V_C = -10$ mV. *B*, *I–V* relationship for the same retinal ganglion cell showing the antagonism of nifedipine (10 $\mu$M) on currents evoked from $V_H = -90$ mV to a variety of test potentials. ●, the currents obtained during application from a pneumatic pipette of bath solution (same composition as in Fig. 1 with 10 mM-$Ba^{2+}$ as the charge carrier). ○, the currents observed during application of a second pneumatic pipette's contents identical to the first except for the addition of 10 $\mu$M-nifedipine. In the presence of nifedipine, the peak current was shifted along the abscissa by +10 mV compared to control. The patch pipette solution contained (in mM): CsCl, 120; TEA-Cl, 20; HEPES, 10; EGTA, 1·5; ATP, 3; GTP, 1; $CaCl_2$, 1; $MgCl_2$, 1; pH 7·2.

an increase in the whole-cell $Ca^{2+}$ current. These agonist qualities are especially evident at hyperpolarized holding potentials (Hess, Lansman & Tsien, 1984). As shown in Fig. 4, in retinal ganglion cells 5 $\mu$M-Bay K 8644 produced activation of the prolonged component of calcium current at more hyperpolarized test potentials without greatly affecting the transient current ($n = 6$). In most cells (four out of six) the peak calcium current was also increased with the addition of 5 $\mu$M-Bay K 8644. In contrast, with higher concentrations (e.g. 20 $\mu$M) an increase in peak calcium current was no longer consistently observed ($n = 7$; not illustrated). Thus, with regard to dihydropyridines the prolonged component of $Ca^{2+}$ current in retinal ganglion cells appears to have a somewhat similar pharmacology to that reported for the L-type current in DRG neurones or in cardiac cells.

*Unitary properties of channels underlying the calcium current*

Single-channel recordings were obtained in the cell-attached mode from twenty-eight patches on retinal ganglion cell neurones. At least two types of channel with distinct behaviour were observed (Fig. 5). The first type of channel was of small amplitude and in general opened near the beginning of depolarizing voltage steps. Often only a single opening occurred; but even when multiple events were visualized, the activity had usually completely subsided within 30 ms of the onset of a voltage step to −30 mV, as seen in Fig. 5*A*. The second type of channel had a larger unitary conductance, displayed activity throughout a step of 100 ms or more, and often had prolonged openings in addition to brief ones (Fig. 5*B*). The amplitude and kinetics of the first kind of channel activity in the present study appeared to correspond to the T-type $Ca^{2+}$ channel, and the second kind to some extent, with the channel

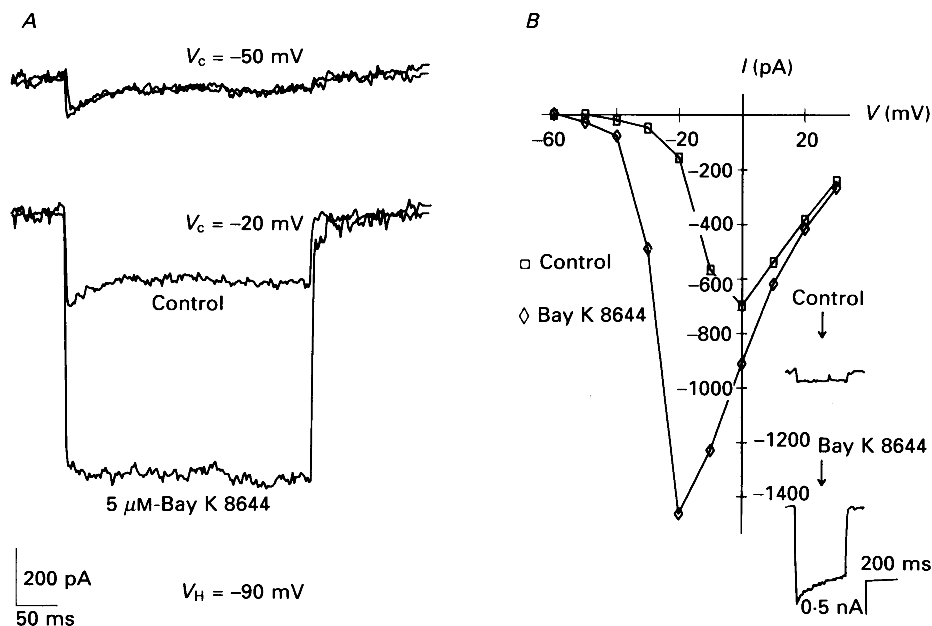


Fig. 4. Sensitivity of the prolonged component of calcium current in retinal ganglion cells to the dihydropyridine agonist Bay K 8644. *A*, during whole-cell recording, test potentials ($V_C$) were applied to $-50$ or $-20$ mV from a holding potential ($V_H$) of $-90$ mV. With $Ba^{2+}$ as the charge carrier, 'control' currents were evoked at each potential. Bay K 8644 (5 μM) was then applied for 30 s with no effect on the transient current observed with a step to $-50$ mV (control and treated records superimpose in the top pair of traces). In contrast, enhancement of the prolonged component of current was manifest during a test pulse to $-20$ mV in the presence of Bay K 8644 (lower pair of traces). *B*, *I–V* characteristic for a family of calcium currents in another cell obtained from $V_H = -90$ mV before and after the addition of Bay K 8644 (5 μM). In this representative case, after drug treatment the prolonged component of current was activated at more negative command potentials and the maximal current was increased. The inset shows two of the current records obtained in the presence and absence of Bay K 8644 with $V_C = -20$ mV. For these experiments retinal ganglion cells were locally microperfused from a pneumatic pipette with the bath solution listed in Fig. 1 with 10 mM-$Ba^{2+}$ as the charge carrier or by a second pneumatic pipette with the same solution plus 5 μM-Bay K 8644. The patch pipettes contained (in mM): CsCl, 120; TEA-Cl, 20; $MgCl_2$, 1; ATP, 3; BAPTA, 5; HEPES, 10; pH 7·2.

underlying a sustained calcium current, as detailed previously in other tissues (Fox 1987*a*, *b*; Hagiwara *et al.* 1988). These findings may not account for the slowly decaying phase of the current observed in many whole-cell recordings (e.g. in Fig. 1*B*). One possible explanation for the slowly decaying phase in the whole-cell recordings is a voltage- or current-dependent inactivation that is absent under the conditions of cell-attached patch recording with a high concentration of $Ba^{2+}$ in the pipette as the charge carrier.

Figure 5*C* shows the unitary *i–V* relations for the two types of channel. One example of an amplitude histogram used to measure the magnitude of the current for the larger channel is illustrated in the inset. The slope conductances associated with the small and large channels at 35 °C when 96 mM-$Ba^{2+}$ was in the patch pipette were $\sim$ 8 and $\sim$ 20 pS, respectively.

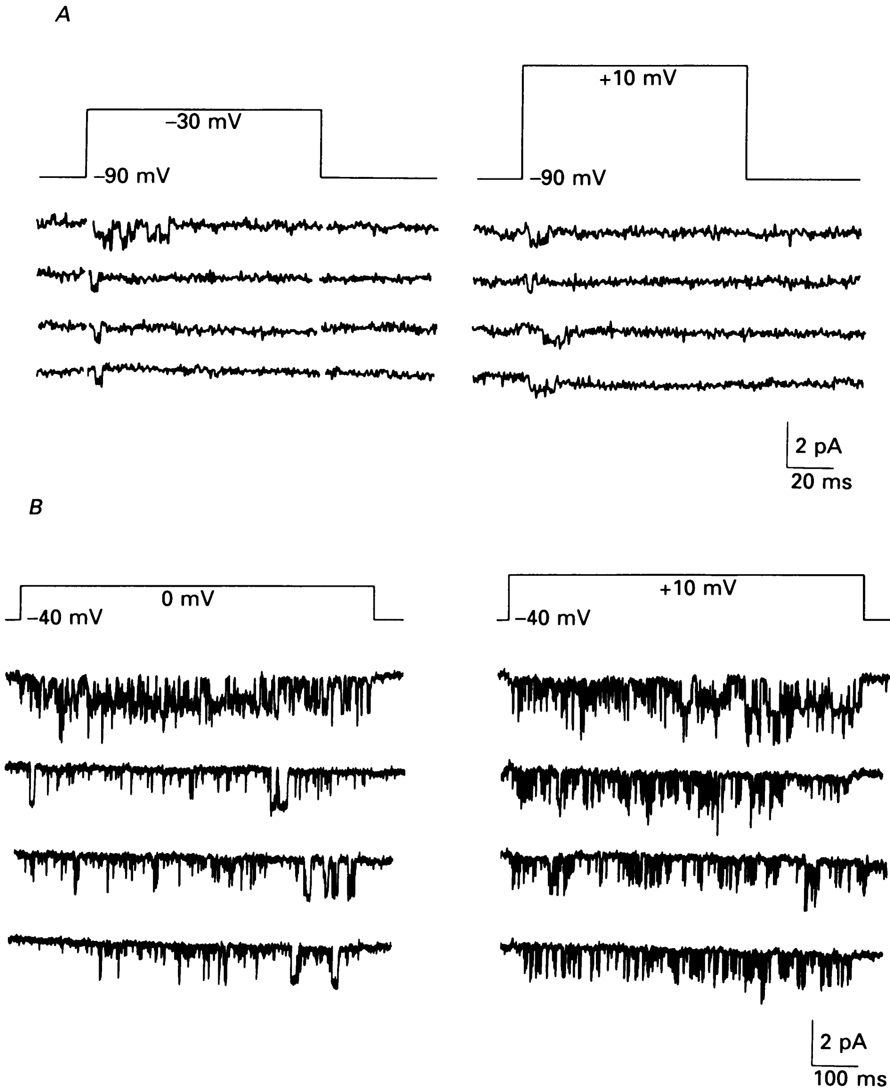


Fig. 5 (*A*,*B*). For legend see p. 390.

*Rapidly reversible inhibition of both components of calcium current by ω-CgTX VIA*

Purified toxin ω-CgTX VIA was the kind gift of Dr B. M. Olivera (University of Utah). Figure 6*A* shows that, unlike the effect reported in other nervous tissues, the application of 10 μM-ω-CgTX VIA from a pneumatic pipette produced a rapidly reversible inhibition of whole-cell $Ca^{2+}$ current, and all components were affected. Within seconds of addition, the toxin produced a 30–85% blockade of both the transient and prolonged contributions to the $Ca^{2+}$ current. This effect was completely reversible within approximately 15 s (the shortest interval examined).

The reduction of the amplitude of the peak current was not due only to a massive blockade of the prolonged component. This is seen by examining the blockade in

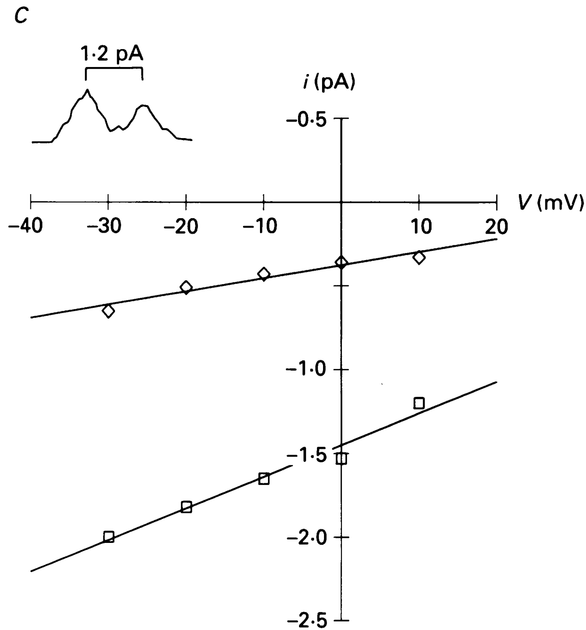


Fig. 5. Single $Ca^{2+}$ channel recordings in rat in retinal ganglion cells. Current traces from two different cell-attached patches are illustrated, each at two different command voltages. The patch potential is shown above the current traces. *A*, transient-type $Ca^{2+}$ channel. For this type of channel, activity had usually ceased within 20–30 ms of the beginning of the voltage step, as also observed in the whole-cell recordings. This particular patch had at least two of these small channels, and no larger channels were activated. *B*, larger, sustained-type $Ca^{2+}$ channels. More than one of the larger types of channel was present in this patch and double openings were observed fairly frequently. The larger channel type was studied in isolation by holding the membrane at a relatively depolarized potential (−40 mV). With greater temporal resolution, the very brief, smaller events in these traces appear to represent incompletely resolved openings of the larger channel. Standard techniques for cell-attached patch recording were used with the cell membrane potential brought to zero (Fox *et al.* 1987*b*) by the bath solution, which contained (in mM): potassium aspartate, 140; K-EGTA, 10; $MgCl_2$, 1; HEPES, 10; pH 7·2 with KOH. The pipette solution contained: 96–110 mM-$BaCl_2$, 10 mM-HEPES (occasionally with 1 μM-TTX, for example, in *A*), pH 7·2 with TEA-OH. *C*, unitary current–voltage (*i*–*V*) relations for the small and large channels. Data were pooled from six patches for the smaller channels (◇) and five patches for the larger channels (□). In the inset, a representative current histogram, from which the data points were obtained, is shown for the larger channel at $V_C$ = +10 mV (as in *B*, above). The straight lines were fitted by a least-squares method to the data with slopes ($\gamma$) and coefficients of correlation ($r^2$) as follows: smaller channel, $\gamma$ = 8 pS and $r^2$ = 0·97; larger channel, $\gamma$ = 20 pS and $r^2$ = 0·99.

detail. In Fig. 6*B* the transient and prolonged portions of the current are examined at higher resolution to emphasize that both are affected by ω-CgTX VIA. Furthermore, Fig. 6*C* and *D* show that the degree of block is voltage-dependent. In Fig. 6*D* the diagram illustrates that for both the transient and prolonged components the effectiveness of the blockade increased with more depolarizing command steps. At more negative command potentials ($V_C < -20$ mV), where the transient current contributed relatively more to the total current, the prolonged current was blocked

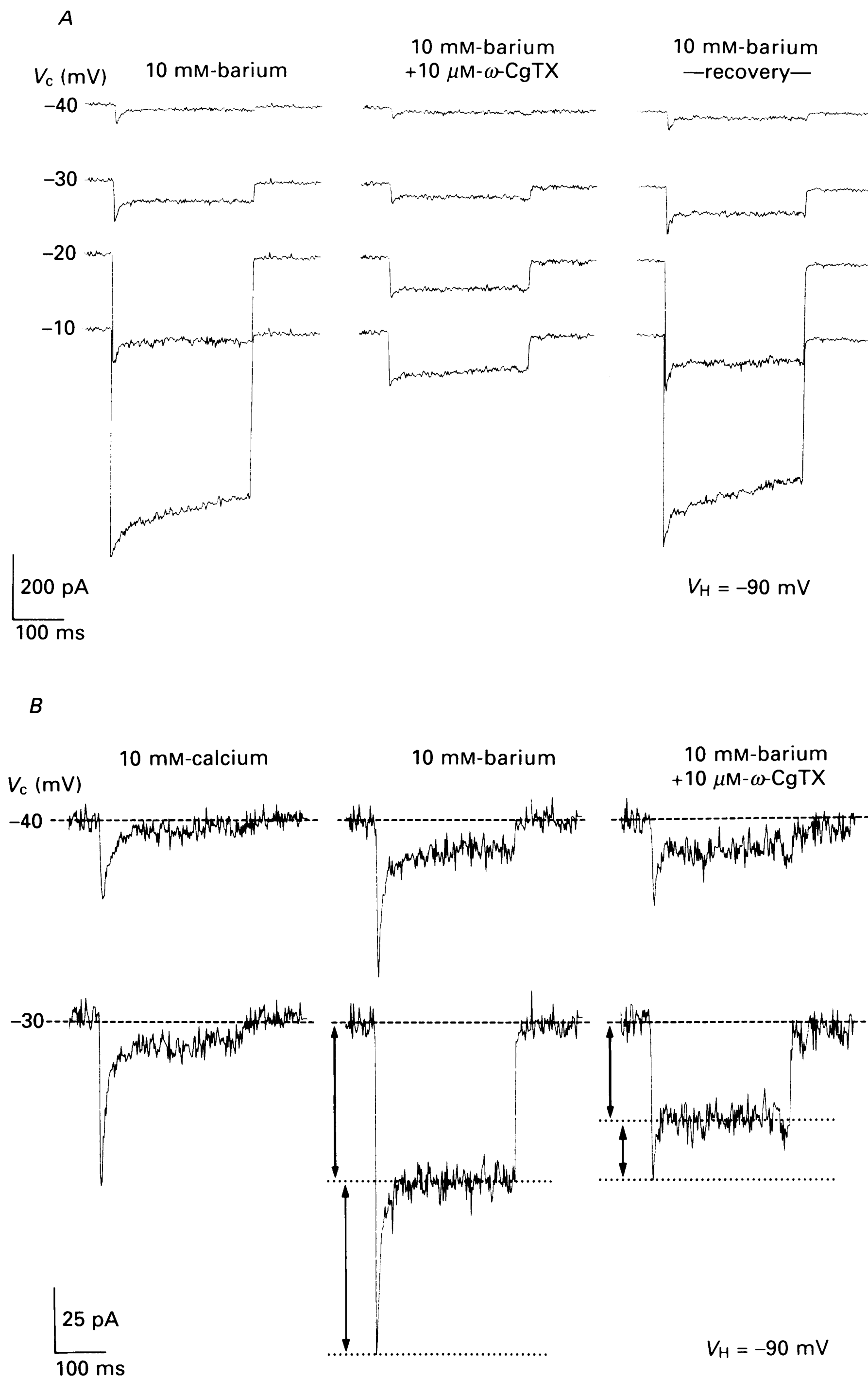


Fig. 6 (*A*, *B*). For legend see p. 392.

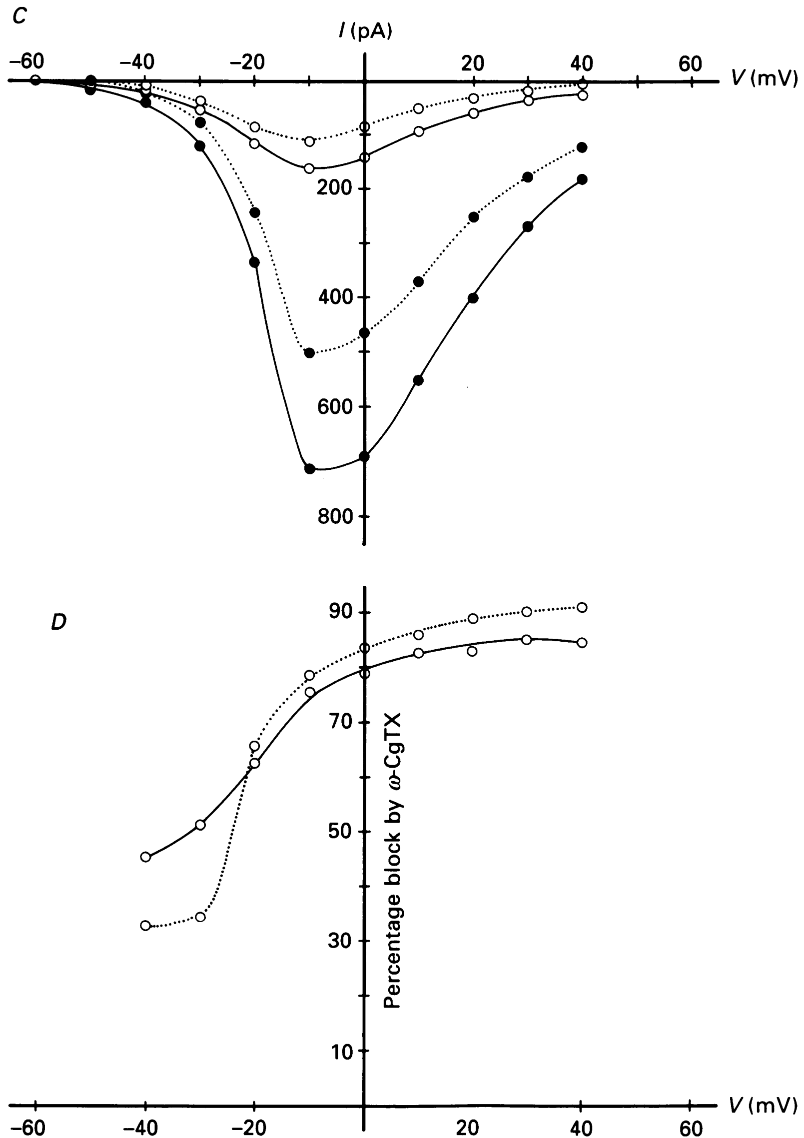


Fig. 6. Transitory effect of ω-CgTX VIA on both components of calcium current in rat retinal ganglion cells. *A*, whole-cell currents with $Ba^{2+}$ as the charge carrier obtained before, during, and after the addition of 10 μM-ω-CgTX VIA. The toxin, mixed in bath solution, was applied by pressure ejection from a microcapillary pipette. Recovery was complete within 15 s of the cessation of toxin addition. *B*, comparison of the current components at a higher level of resolution for two command potentials ($V_C$). For comparison, the left-hand set of traces were obtained with $Ca^{2+}$ rather than $Ba^{2+}$ as the charge carrier. In the middle set of traces note the increase in the prolonged current by equimolar substiution of $Ca^{2+}$ with $Ba^{2+}$ concomitant with very little effect on the transient current (cf. Fig. 2*C*). Arrows represent amplitudes of the sustained and transient components at $V_C = -30$ mV in the presence and absence of ω-CgTX VIA. Note the change in the ratio of the amplitudes of the transient to sustained components with the addition of the toxin. *C*, *I–V* curves of the peak transient (continuous lines) and prolonged currents (dotted lines) of the cell in *A* before (●) and after (○) ω-conotoxin

to a somewhat greater extent than the transient component (50 *versus* 30% block). In contrast, above −20 mV, the degree of block engendered by the toxin was slightly greater for the transient current (85–90% block at $V_C = 0$ to +40 mV) than for the prolonged component (80–85% block over the same voltage range).

## DISCUSSION

This paper presents the first voltage-clamp evidence in favour of the hypothesis that there exists more than one component of calcium current in a clearly identified cell type cultured from the *post-natal* mammalian central nervous system. Whole-cell recordings from rat retinal ganglion cells revealed that there are at least two contributions to the calcium current in this preparation. Moreover, to date, single-channel recordings have yielded evidence for at least two types of $Ca^{2+}$ channel in this system. One type appears to correspond fairly closely with the T or transient component of calcium current studied previously by others (see Introduction for references). This component has a threshold for activation at low voltages (−40 to −50 mV from holding potentials negative to −80 mV), inactivates with a time constant of 10–30 ms, and has a small single-channel conductance of about 8 pS. In whole-cell recordings substitution of equimolar $Ba^{2+}$ for $Ca^{2+}$ results in transient currents of nearly equal amplitude. A more prolonged contribution to the calcium current in whole-cell records can be fitted by slowly decaying and sustained kinetic parameters; the slow decay could be secondary to voltage- or current-inactivation of the sustained component of the current. The unitary events underlying the latter current have a single-channel conductance of ~ 20 pS with 96 mM-$Ba^{2+}$ as the charge carrier and may display long open times (mode 2 gating; Hess *et al.* 1984). The agonist dihydropyridine Bay K 8644 (5 μM) resulted in activation of the prolonged component of calcium current (but not the transient component) at a more hyperpolarized level and increased the peak current in nearly every ganglion cell tested; these findings are not dissimilar to agonist dihydropyridine effects on L-type current in other preparations (Schramm, Thomas, Towart & Franckowiak, 1983; Hess *et al.* 1984). In addition, the antagonist dihydropyridines nifedipine and nimodipine (1–10 μM), inhibit the prolonged component of calcium current in retinal ganglion cells in a manner similar to the block they exert on the L-current in other systems.

A major point of the present investigation concerns the action of the toxin ω-CgTX VIA on the calcium current of a post-natal, mammalian central neurone such as the type studied here. This toxin was originally isolated from the marine fish-hunting snail *Conus geographus* by B. M. Olivera and colleagues (Olivera, Gray, Zeikus, McIntosh, Varga, Rivier, de Santos & Cruz, 1985; Cruz & Olivera, 1986.). In chick DRG neurones ω-CgTX VIA has been reported to depress the T-type of $Ca^{2+}$

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block. *D*, voltage dependence of the degree of blockade of $Ca^{2+}$ current by ω-CgTX VIA for the same retinal ganglion cell as in *A*. The continuous and dotted lines represent the blockade of the prolonged and peak transient currents, respectively. The bath solution was the same as in Fig. 1 with 10 mM-$Ba^{2+}$ as the charge carrier except where indicated that equimolar $Ca^{2+}$ was substituted. The patch pipettes contained (in mM); CsCl, 120; TEA-Cl, 20; $MgCl_2$,1; $CaCl_2$, 1; EGTA, 1·5; ATP, 3; HEPES, 10; pH 7·2 with TEA-OH.

channels in an ephemeral manner whilst producing a more persistent blockade of the other $Ca^{2+}$ channels (N- and L-types affected irreversibly according to McCleskey *et al.* 1987, or N-type affected irreversibly and L-type reversibly in the experiments of Kasai, Aosaki & Fukada, 1987).

The toxin ω-CgTX VIA has been used to study the functional significance of particular $Ca^{2+}$ channel subtypes on neurotransmitter release. The interpretation of these experiments has been that a particular $Ca^{2+}$ channel subtype is involved in neurotransmitter release. This conclusion is based on the findings that ω-CgTX VIA acts differentially on the disparate types of $Ca^{2+}$ channel (for example, not affecting the T-type) and that the action of the toxin is long-lasting (as found by McCleskey *et al.* 1987). Quite to the contrary of those results, we find that in rat retinal ganglion cells both the transient and prolonged components of calcium current are inhibited by ω-CgTX VIA and that this blockade is rapidly reversible with complete recovery occurring within seconds. Obviously, from our data this fraction of ω-conotoxin cannot be used as a pharmacological agent to distinguish between the types of $Ca^{2+}$ channel present in rodent retinal ganglion cells. Thus, in our preparation ω-CgTX VIA acts in a manner distinct from that reported in other tissues and serves to distinguish the prolonged component of calcium current in retinal ganglion cells from the L-type current in, for example, chick DRG neurones. An alternative explanation for our results would be that the L-type current in retinal ganglion cells is similar to that found in other preparations but that a second type of large channel whose main state is of similar conductance (perhaps an 'N' type channel) is sensitive to ω-CgTX VIA (Plummer, Logothetis & Hess, 1989). In any event, these findings highlight the need for extreme caution in interpreting the effects of these reagents on transmitter release or on other functions of central neurones in the absence of a thorough pharmacological knowledge of the various components of calcium current in the particular system under study.

Taken together with the data of others, our results suggest that the various types of $Ca^{2+}$ channel in different species, and even in disparate neurones of the same species, may resemble one another to some extent but have, in addition, evolved characteristics peculiar to the tissue. Along these lines, molecular evidence is rapidly accumulating that each ion channel and receptor–channel complex, while displaying significant homology, contains variation in the structure of subunits located in disparate areas within a single organism (for example, for a description of the heterogeneity of the neural nicotinic receptor–channel complex, see Wada, Ballivet, Boulter, Connolly, Wada, Deneris, Swanson, Heinemann & Patrick, 1988). Our electrophysiological findings predict a similar heterogeneity for calcium channels in different tissues. Presumably, these variations reflect some subtlety in the physiological functions that distinguish these tissues, especially in the mammalian central nervous system, but these distinctions are as yet unclear. In addition, these tissue-specific peculiarities of ion channels (Lester, 1988) offer the hope that clinically useful tissue-specific channel antagonists may be constructed from a detailed knowledge of the variation in each type of channel's structure.

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