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The cisternal organelle as a Ca^{2+}-storing compartment associated with GABAergic synapses in the axon initial segment of hippocampal pyramidal neurones

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Abstract The axon initial segment of cortical principal neurones contains an organelle consisting of two to four stacks of flat, membrane-delineated cisternae alternating with electron-dense, fibrillar material. These cisternal organelles are situated predominantly close to the synaptic junctions of GABAergic axo-axonic cell terminals. To examine the possibility that the cisternal organelle is involved in Ca^{2+} sequestration, we tested for the presence of Ca^{2+}-ATPase in the cisternal organelles of pyramidal cell axons in the CA1 and CA3 regions of the hippocampus. Electron microscopic immunocytochemistry using antibodies to muscle sarcoplasmic reticulum ATPase revealed immunoreactivity associated with cisternal organelle membranes. The localisation of Ca^{2+}-ATPase in cisternal organelles was also confirmed by enzyme cytochemistry, which produced reaction product in the lumen of the cisternae. These experiments provide evidence for the presence of a Ca^{2+} pump in the cisternal organelle membrane, which may play a role in the sequestration and release of Ca^{2+}. Cisternal organelles are very closely aligned to the axolemma and the outermost cisternal membrane is connected to the plasma membrane by periodic electron-dense bridges as detected in electron micrographs. It is suggested that the interface acts as a voltage sensor, releasing Ca^{2+} from cisternal organelles upon depolarisation of the axon initial segment, in a manner similar to the sarcoplasmic reticulum of skeletal muscle. The increase in intra-axonal Ca^{2+} may regulate the $GABA_A$ receptors associated with the axo-axonic cell synapses, and could affect the excitability of pyramidal cells.

Key words Calcium · Calcium-activated ATP-ase GABA · Immunocytochemistry · Synapse

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Introduction

The axon initial segment (AIS) of cortical and hippocampal principal neurones contains a structure called the cisternal organelle, consisting of stacks of membrane-delineated flat stacks interspersed with an electron-dense granular material (Kosaka 1980; Palay et al. 1968; Peters et al. 1968; Sloper and Powell 1979; Somogyi et al. 1983b). The axon initial segment and these organelles can be recognised in the electron microscope on the basis of their fine structural characteristics (Peters et al. 1991). The cisternal organelle is similar structurally, and perhaps also functionally, to the spine apparatus which has been shown to accumulate and store calcium (Andrews et al. 1988), and has inositol 1,4,5-trisphosphate receptor-activated Ca^{2+} channels in its membrane (Satoh et al. 1990; Takei et al. 1992). The spine apparatus therefore is thought to be involved in the sequestration of calcium entering the cell and/or in the release of endogenous calcium mediated by second messengers (see review, Rossier and Putney 1992). Nothing is known about the chemistry of the analogous organelle in the axon initial segment of pyramidal cells, which have another feature unique to cortex; they receive dense GABAergic input from the so called axo-axonic cells (Somogyi 1977; Somogyi et al. 1983a, b; 1985).

Is it possible that the two unique features of pyramidal cell AISs, namely the presence of cisternal organelles and the dense GABAergic input, are related? The identification of the molecular structure of the cisternal organelle and the AIS would help to answer this question. As a first step, we began testing the cisternal organelle for the presence of markers indicating Ca^{2+} sequestration, such as the enzyme Ca^{2+}-ATPase, which is responsible for pumping calcium across membranes against the electrochemical gradient (see Grover and Khan 1992). Electron microscopic immunocytochemistry and enzyme histochemistry were used to localise Ca^{2+}-ATPase.

Materials and methods

Immunocytochemistry

Preparation of animals and tissue

Six adult female or male Wistar rats (100–400 g) were deeply anaesthetised with sodium pentobarbital (150 mg/kg, i.p.). They were perfused through the aorta for 10–30 min with NaCl solution (0.9% for 1 min) followed by ice-cold fixative containing 4% paraformaldehyde, 0.025% glutaraldehyde and approximately 0.2% picric acid (Somogyi and Takagi 1982) made up in 0.1 M phosphate buffer (PB, pH 7.2).

Immunoreaction

The rabbit polyclonal antiserum (designated EM-3) was raised against sarcoplasmic reticulum Ca^{2+}-ATPase, which was purified from rat gastrocnemius muscle (Molnár et al. 1990). Previous reports on this antiserum established its specificity to isoenzymes of several sarcoplasmic and endoplasmic Ca^{2+}-ATPase, but it has not been found to react with the plasma membrane, calcium pump proteins (Molnár et al. 1990, 1992; Sarkadi et al. 1988). Partial proteolysis and immunoblot analysis revealed antiserum reaction with all major fragments of the sarcoplasmic reticulum Ca^{2+}-ATPase (Molnár et al. 1990). Affinity purified antibodies were also used in some immunochemical experiments, and they gave identical and very specific results with the appropriately diluted full serum (E. Molnár, unpublished observations). The antiserum was specific for Ca^{2+}-ATPase and dit not react with Na^+, K^+-ATPase or H^+, K^+-ATPase (Molnár et al. 1992). The immunoreactivity of Ca^{2+}-ATPase was preserved after fixation of tissue samples (Krenács et al. 1989).

Immunocytochemical procedures were carried out as described earlier (Molnár et al. 1993). Antiserum to the sarcoplasmic reticulum Ca^{2+}-ATPase was used at a final dilution of 1:1 000. Primary antibodies were localised by immunoperoxidase reaction using the avidin biotinylated horseradish peroxidase complex method. Sections were incubated for 1 h in a mixture of biotinylated goat anti-rabbit IgG (diluted 1:50, Vector Labs, Peterborough, England), followed by washing and incubation in avidin-biotinylated horseradish peroxidase complex solution (Vector). Peroxidase was revealed using 3,3' diaminobenzidine tetrahydrochloride (DAB, 0.015%) dissolved in 50 mM Tris-HCl (pH 7.2) and H_2O_2 (0.01%). Sections were routinely processed either for light or electron microscopic examination (Molnár et al. 1993).

In control incubations, the primary antiserum was replaced by normal rabbit serum at the same dilution. Selective distribution of labelling was not observed in the controls, indicating that the reactions described in this paper are due to the primary antiserum to Ca^{2+}-ATPase.

Enzyme cytochemistry for Ca^{2+}-ATPase

Five Wistar rats were perfused via the heart with 4% paraformaldehyde solution in 0.1 M cacodylate buffer (pH 7.2) for 40 min at room temperature. After removing the brain, 70-μm-thick sections were cut from the hippocampus and cerebellum using a vibratome. The sections were washed overnight in cold 0.1 M cacodylate buffer, followed by 30 min washing in 250 mM glycine-NaOH buffer (pH 9.0). Incubation for Ca^{2+}-ATPase was carried out in a water bath at 37° C for 30–40 min according to Ando et al. (1981). The incubation medium consisted of 250 mM glycine-NaOH buffer (pH 9.0), 3 mM ATP (Sigma), 10 mM $CaCl_2$, 2 mM lead citrate and 10 mM levamisole (L[-]-2,3,5,6,-Tetrahydro-6-phenylimidazo[2,1-b]-thiazole, Sigma) for the inhibition of non-specific alkaline phosphatase. After incubation, the sections were washed for 5 min in 0.1 M cacodylate buffer (pH 7.2). Postfixation was carried out in 2% OsO_4 buffered with 0.1 M cacodylate buffer to pH 7.2. Staining with 1% uranyl acetate was performed for 30 min during dehydration in the 70% alcohol stage. Samples were embedded in epoxy resin (Durcupan ACM, Fluka).

Controls included omitting (a) ATP, (b) Ca^{2+} or (c) both ATP and Ca^{2+} from the incubation medium. Under control conditions no significant electron dense deposit was observed in the tissue indicating that the electron-dense deposit obtained with the full medium is due to Ca^{2+}-ATPase. In order to increase the tissue ATP content, in some experiments, pre-incubation was applied in 3 mM ATP-Na dissolved in 250 mM glycine-NaOH buffer (pH 9.0) prior to changing to the full incubation medium. No consistent improvement was observed in the reaction in intracellular organelles. In some experiments, 0.5% or 5% DMSO was added to the pre-incubation solution to increase the penetration of ATP, but no significant improvement was observed.

Results

Fine structural organisation of the cisternal organelle in the IS of pyramidal neurones

Axon initial segments are recognised on the basis of the electron-dense, membrane undercoating on the cytoplasmic face of the axonal membrane (Fig. 1A) and by the presence of fasciculated microtubules (Peters et al. 1991). As described in earlier studies, the axon initial segments of cortical principal cells, i.e. pyramidal cells, spiny stellate cells and dentate granule cells also frequently contain cisternal organelles (Kosaka 1980; Palay et al. 1968; Peters et al. 1968; Sloper and Powell 1979; Somogyi et al. 1983b). In conventional electron microscopic material, cisternal organelles consist of two to four stacks of flat, membrane-delineated cisternae alternating with electron-dense fibrillar material which is separated from the cisternal membrane by an electron-lucent gap on both sides (Fig. 1). The lumen of individual cisternae is variable and can be as narrow as about 5–10 nm (Fig. 1B).

Cisternal organelles are usually situated near GABAergic synapses at the periphery of the axoplasm, but they extend beyond the area of any given synapse. Since cisternal organelles are larger structures than synapses, the two may not be present in all single electron microscopic sections, even if they were associated. Since AISs in the rat hippocampus are densely, but unevenly, covered by synapses, complete three dimensional reconstruction would be necessary to establish quantitatively the association between cisternal organelles and synaptic junctions. This was not carried out in the present study, nevertheless it emerged that it is much easier to find cisternal organelles near synapses than in non-synaptic regions of the AIS. The outermost cisternal membrane forms close associations with the plasma membrane. The postsynaptic junctional area of the axonal membrane is not contacted by the cisternal organelles which often lie just to the side of the synapse (Fig. 1). In longitudinal sections of the axon, when cisternal organelles can be seen along their long axis the junctions with the plasma membrane are found periodically (Fig. 1A). The distance between the outermost cisternal membrane and the axolemma is about 10–20 nm and, in some planes of sectioning, periodic elec-

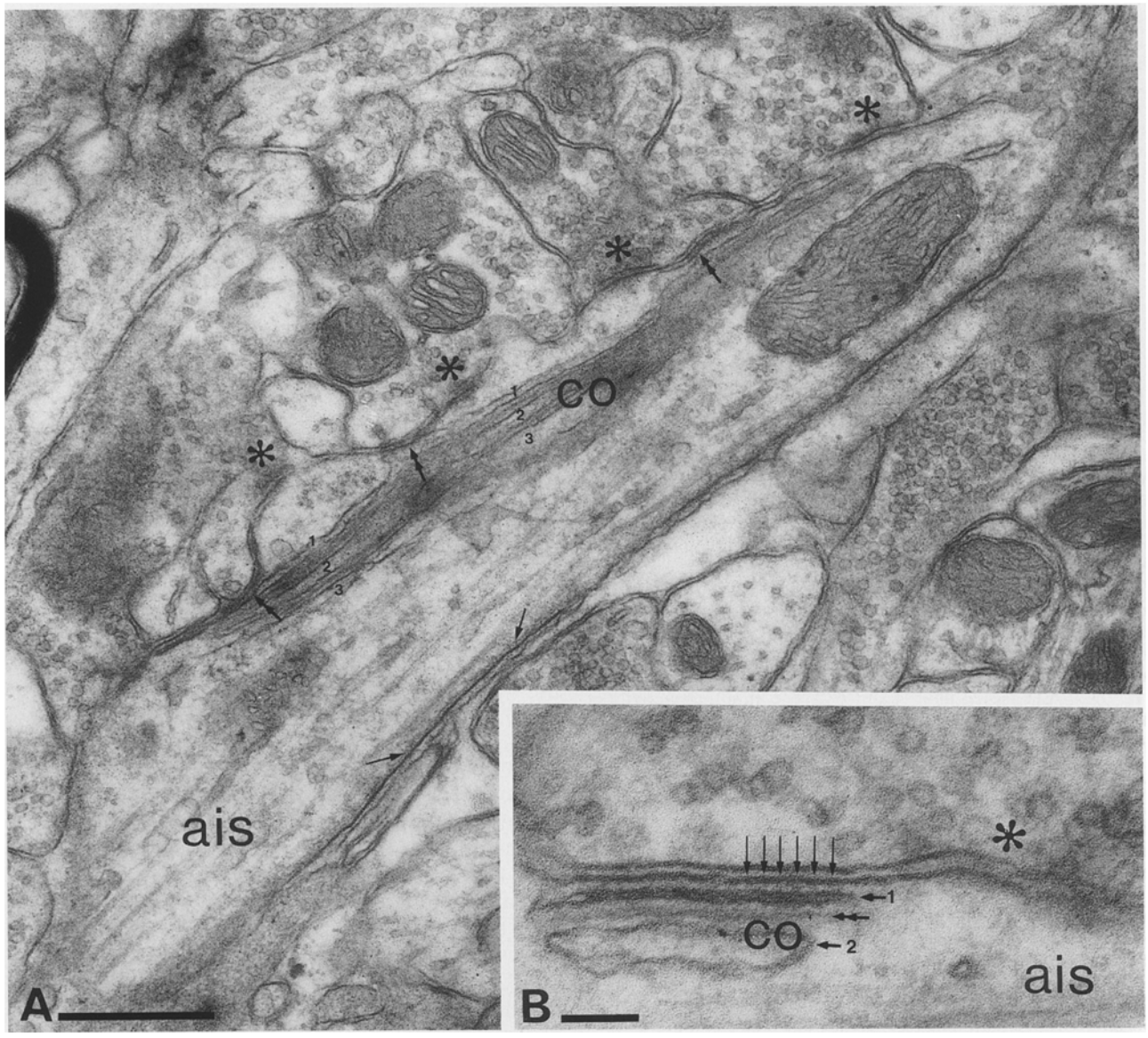

Fig. 1 Cisternal organelles (co) shown in longitudinal (**A**) and cross-sections (**B**) in the axon initial segment (ais) of hippocampal pyramidal neurones in the CA1 area. In **A** the organelle consists of three cisternae (1–3) and two intervening electron dense fibrillar bands. Notice repeated close association (*double arrows*) of the cisternal organelle in **A** with the plasma membrane in the vicinity of synaptic junctions (*asterisks*). Periodic bridges (*vertical arrows* in **B**) connect the outermost cisternal membrane to the plasma membrane. In this example, the two membranes of one (1) of two cisternae (1, 2) are partly fused. The intercisternal, electron-dense band is indicated by *double arrow* in **B**. The axon initial segment can be identified by the presence of the electron-dense membrane undercoating (e.g. between single arrows in **A**). *Scale bars A* 0.5 μm, *B* 0.1 μm

tron-dense bridges can be seen joining the two membranes (Fig. 1B). In contrast to cisternal stacks in the somata and dendrites of Purkinje cells (Rusakov et al. 1993), the bridges in the initial segment of pyramidal cells are not present between cisternae instead, as mentioned above, they are separated by electron-dense fibrillar material.

Cisternal organelles are almost always present in the axonal spines which are found frequently on hippocampal principal neurones (Figs. 3, 4). In contrast to dendritic spines, in axonal spines the cisternal organelle membrane forms close association with the plasma membrane (Somogyi et al. 1983b, Fig. 8A).

Electron microscopic localization of Ca^{2+}-ATPase immunoreactivity in stratum pyramidale of rat hippocampus

Immunoreactive sites were identified by the electron-dense, immunoperoxidase reaction product. Immunoreactivity to Ca^{2+}-ATPase was evident in somata, dendrites, dendritic spines and axon initial segments of hippocampal neurones, and also in some axon terminals (Figs. 2–4). The immunoperoxidase product was always associated with endomembranes in these structures; such as the Golgi apparatus and the endoplasmic reticulum. Some interneurones displayed particularly strong

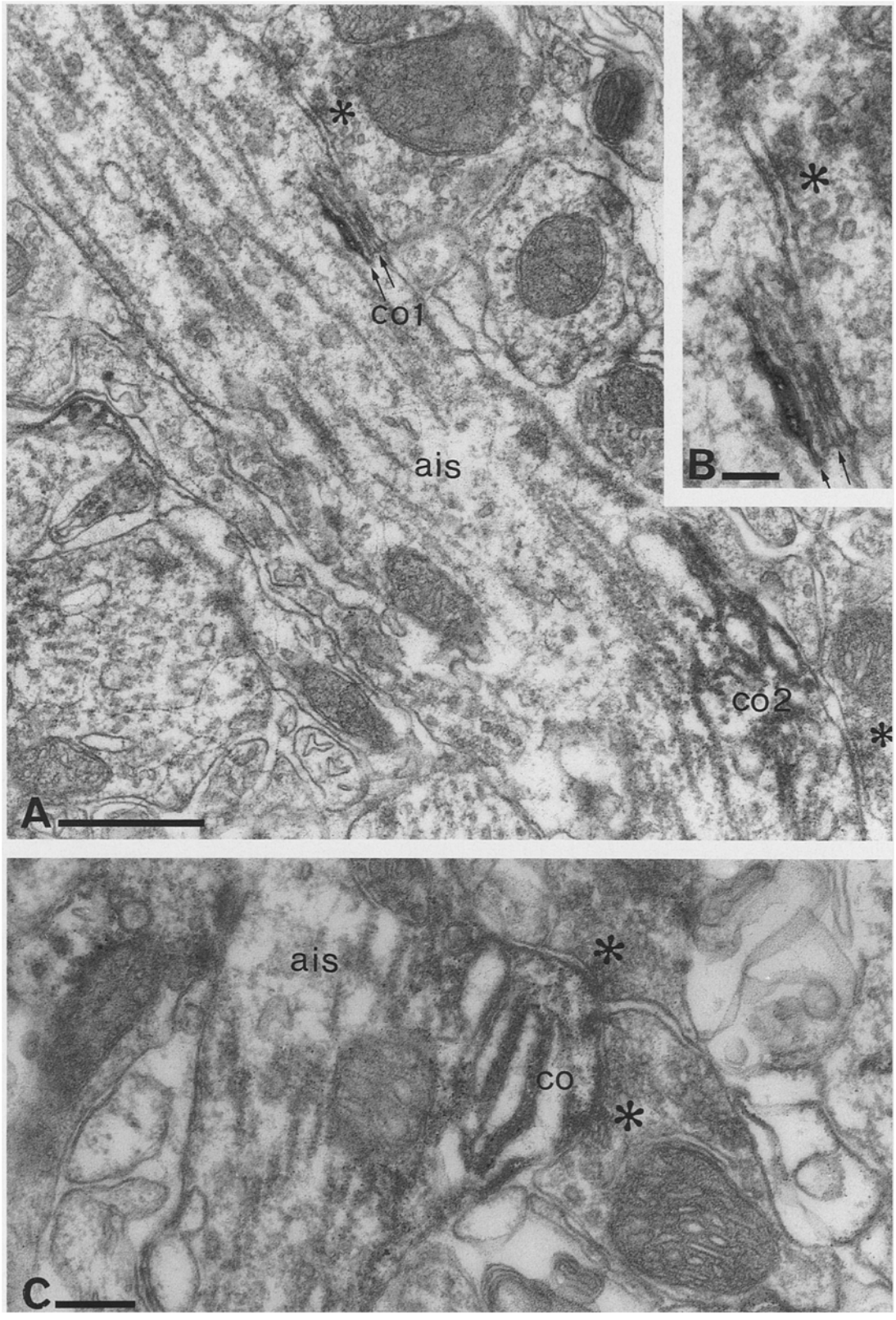

Fig. 2A–C Immunoreactivity for Ca^{2+}-ATPase is associated with cisternal organelles (co) in axon initial segments (ais) as demonstrated by immunoperoxidase reaction. **A** Immunolabelling occurs either in the lumen (co1) or in the pericisternal space (co2). **B** The close association of co1 with a synaptic junction (*asterisk*) is shown at higher magnification. Only the inner cisternal lumen contains reaction endproduct. **C** An axonal protrusion containing an immunopositive cisternal organelle (co) receives two synaptic junctions (*asterisks*). *Scale bars A* 0.5 μm, *B* 0.1 μm, *C* 0.2 μm

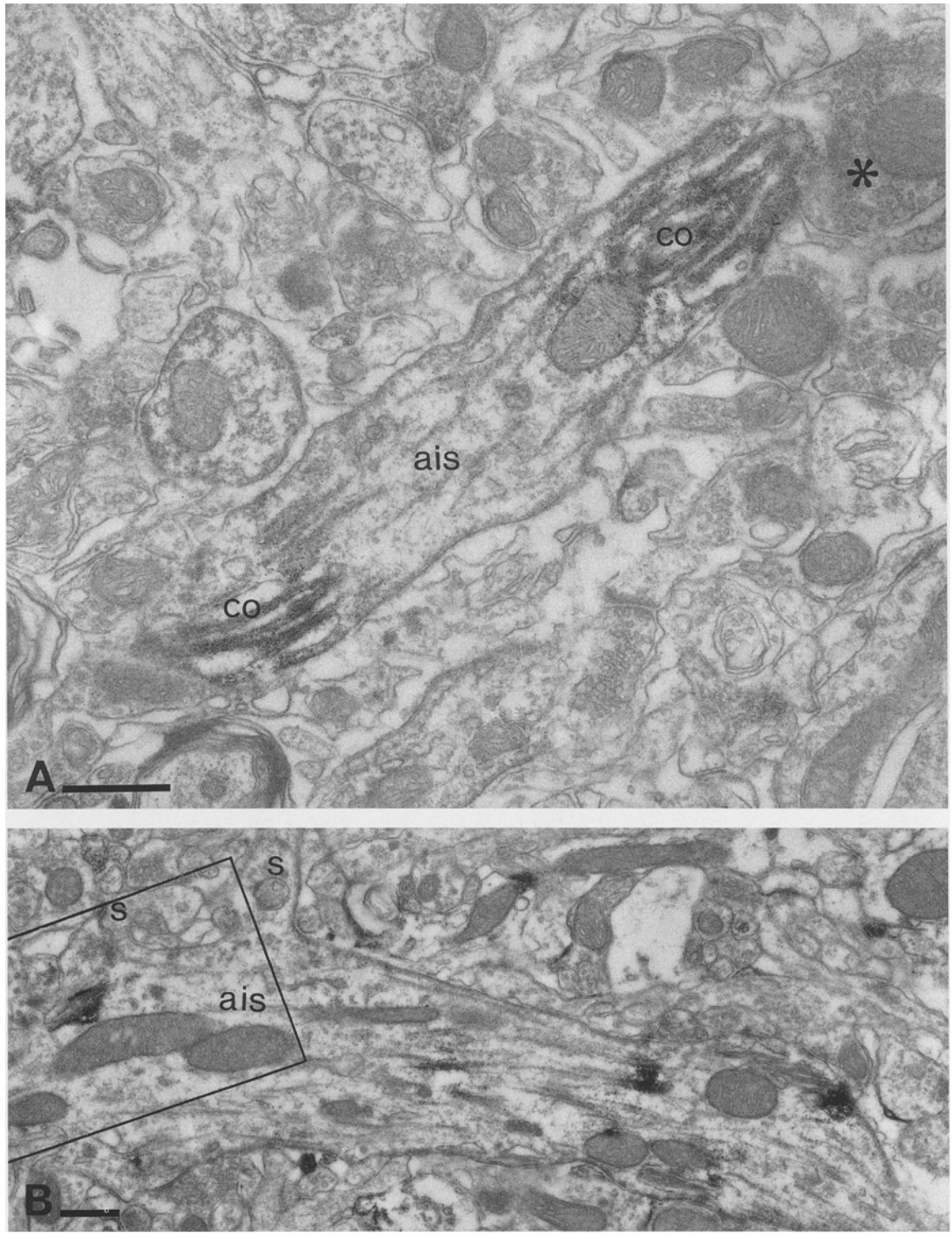

Fig. 3 Immunocytochemical localization of Ca^{2+}-ATPase in the axon initial segments (ais) of pyramidal neurones. **A** Pericisternal immunolabelling is prominent in two cisternal organelles (co), one of them adjacent to a synaptic bouton (*asterisk*). **B** Immunolabelling in an axon initial segment emitting spines (s). Framed area is shown in Fig. 4. at higher magnification. Labelled processes are also found in the neuropil. *Scale bars* 0.5 μm

immunoreactivity (not illustrated). As one of the positive controls, cerebellar Purkinje cells were also analysed in the electron microscope, since the localisation of Ca^{2+}-ATPase has been studied extensively in these cells (e.g. Plessers et al. 1991; Takei et al. 1992). Strong immunoreactivity was associated with the endoplasmic reticulum (not illustrated), supporting the suitability of this antiserum for the localisation of vesicular Ca^{2+}-ATPase.

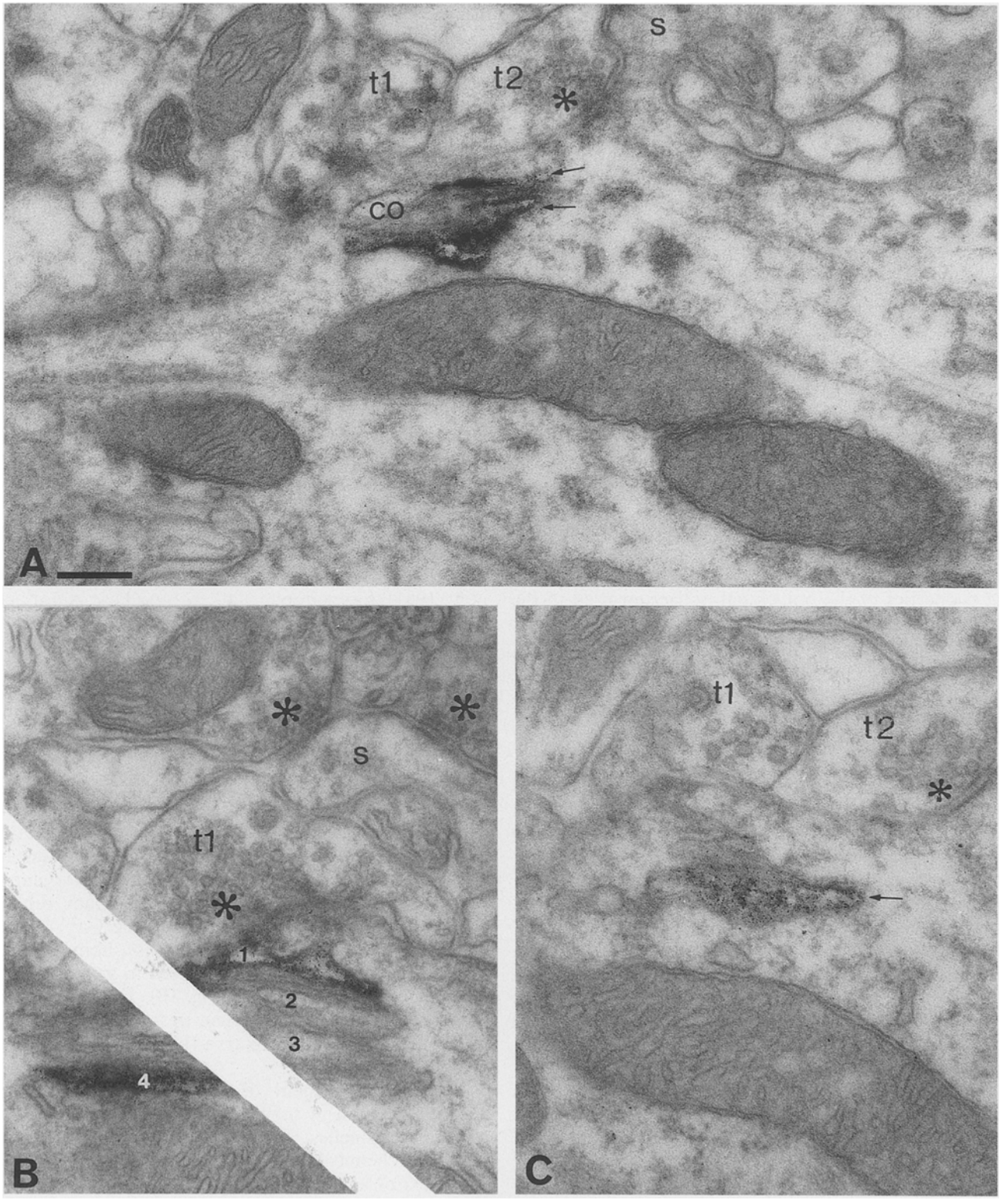

Fig. 4A–C Serial sections of a cisternal organelle, showing intracisternal Ca^{2+}-ATPase immunoreactivity in the two outermost (1, 4 and *arrows*) of four cisternae (1–4). The organelle is adjacent to two synaptic terminals (t1, t2) and a spine which was found to originate from this axon in serial sections (s, see also Fig. 3B). The synaptic junctions of t1 and t2 are cut tangentionally, therefore the synaptic specialisations appear more extensive than in cross-sections. The synaptic specialisation of t2 in C (*asterisk*) is clearly of the type 2. The section shown in **B** had a folding (*white band*), but this does not affect the identification of the organelle. *Scale bar A–C* 0.2 μm

In the following, we focus on the axon initial segments of pyramidal cells, which are the main objects of this study. Therefore, reactivity in other structures is not illustrated separately and is only mentioned when it helps to interpret reaction in the AIS. No difference was observed between the CA1 and CA3 regions, therefore the results are described together. Immunoreactivity was most frequently localised in association with cisternal organelles (Figs. 2–4). The lumen of cisternae were wider in immunoreacted material than in tissue processed without immunoreaction. In most cases, immunoreaction end-product was distributed between the cisternae leaving the lumen free (Figs. 2A, C, 3A). Since HRP reaction product does not spread through the membrane, this reaction predicts epitope(s) on the cytoplasmic side of the cisternal membrane. In some cases, the lumen of the cisternae contained the reaction product indicating epitope(s) on the luminal side of the cisternal membrane (Figs. 2A, B, 3B, 4). The two types of immunoreactivity were present in the same sections and could also be seen in the same axon (Fig. 2A), and occasionally at the same cisternal organelle. The localisation of the endproduct on both sides of the membrane was also detected at sites other than the axon initial segment, and the distribution of labelling is in line with the properties of the polyclonal antiserum used in the present study, which recognises all major proteolytic peptide fragments of the enzyme (Molnár et al. 1990; Sarkadi et al. 1988). Thus, it is likely that several epitopes, present at both sides of the cisternal membrane, contributed to the electron microscopically detected, immunoreaction product. When the reaction product was located in the cisternal lumen, usually only the outermost cisternae – those closest to and/or furthest from the plasma membrane – showed reaction as detected in serial sections (Fig. 4). Some immunoreactivity was also detected along the microtubules of axons (Fig. 3B).

In the vicinity of all immunoreactive cisternal organelles, the axon received synaptic contacts (Figs. 2–4). These synaptic contacts resembled those type 2 junctions reported earlier (Kosaka 1980; Somogyi et al. 1983a) and shown to be provided by boutons immunopositive for glutamate decarboxylase (Somogyi et al. 1983b) or originate from GABA immunopositive neurones (Somogyi et al. 1985). Boutons on AISs studied in conventional material, with few exemptions (Kosaka 1980), usually contain pleomorphic vesicles. The shape of synaptic vesicles in freeze-thawed, peroxidase-reacted material can be different from those observed in conventional electron microscopic material and boutons containing largely round vesicles were also seen in our sections.

Enzyme cytochemical localisation of Ca^{2+}-ATPase in the stratum pyramidale of rat hippocampus

The method of Ando et al. (1981) has been used extensively at the electron microscopic level in many tissues, including the brain, for the localisation of Ca^{2+}-dependent, ATPase activity (Cohen and Kriho 1991; Maggio et al. 1991; Mata and Fink, 1989; Maxwell et al. 1991; Nasu and Inomata 1990; Soji et al. 1991). The reaction product of the Ca^{2+}-dependent ATP hydrolysis is a highly electron-dense precipitate of lead phosphate which was found both extra- and intracellularly in the hippocampus. The reaction varied greatly with depth in the incubated thick sections, indicating limited penetration of the reagents. The reaction product present in the extracellular space generally consisted of larger clumps than the intracellular product, which was usually finely granulated (Figs. 5–7). It has been suggested that the large amount of reaction product precipitated in extracellular spaces is produced by ecto-ATPase, and not by the plasma membrane Ca^{2+} pump (Kortje et al. 1990), but this possibility should not affect the intracellular Ca^{2+}-ATPase localisation. Confusion with other ATPase activities, not depending on the presence of Ca^{2+}, is unlikely under our reaction conditions (Ando et al. 1981); for example, Na^+, K^+-ATPase is not activated in the absence of K^+.

Readily detectable reaction product appeared in the perikarya, as well as in the processes of neurones. Only a limited number of pyramidal cells showed intracellular reactivity for Ca^{2+}-ATPase probably due to the limited penetration of the substrate. The intracellular enzyme reaction product was localised to membrane delineated spaces of the Golgi apparatus and endoplasmic reticulum (Fig. 5A). Some interneurones and glial cells showed very heavy deposition of a coarse reaction product which filled their cytoplasm and processes, including interneurone dendrites (Fig. 5B). The synaptic vesicles and smooth endoplasmic reticulum tubules showed fine granular endproduct of the Ca^{2+}-dependent ATPase reaction (Fig. 6, 7). Dense, homogeneous reaction product was often observed in synaptic clefts formed by dendritic spines (Fig. 7B). Fine granulated reaction product may also occur in some mitochondria, both in the neuronal perikarya (Fig. 5A) and neuronal processes (Figs. 5–7).

The initial segment of pyramidal axons showed ATPase reaction in tubules of the smooth ER and in cisternal organelles (Figs. 6, 7A). Similar to our immunocytochemical results, not every cistern was evenly positive for Ca^{2+}-dependent ATPase in a given cisternal organelle (Fig. 6, 7A). Sometimes only the outer cisterna contained reaction product (Figs. 7A). The ATPase enzyme reactivity in cisternal organelles was similar to that in the spine apparatus of dendritic spines (Figs. 7B). The lack of reaction in some cisternae or in whole cisternal organelles for Ca^{2+}-ATPase may be due to the poor penetration of ATP through cell membranes.

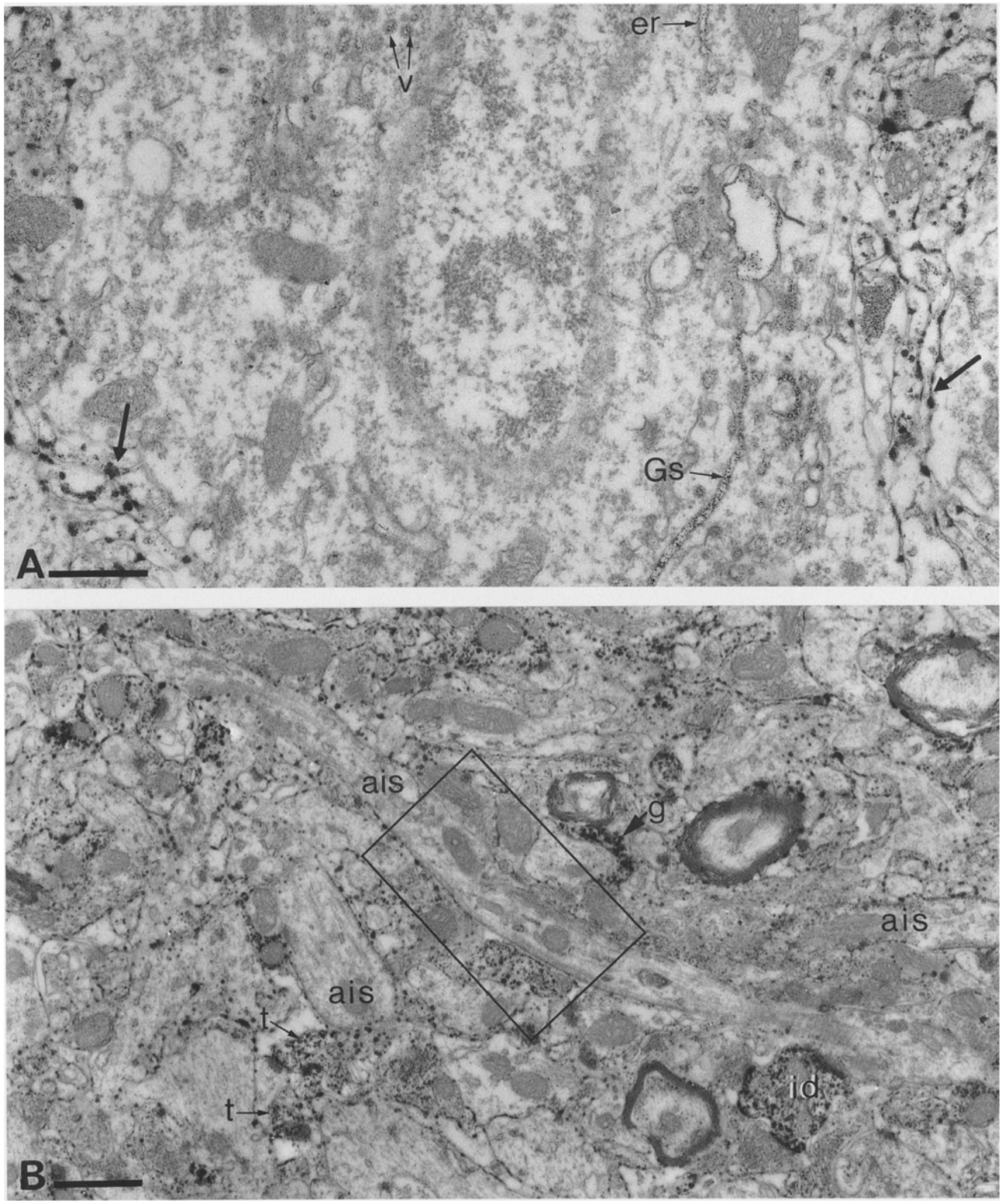

Fig. 5A, B Enzyme cytochemical demonstration of Ca^{2+}-ATPase in the stratum pyramidale. The electron-dense, enzyme reaction end-product is lead phosphate. **A** Strong, patchy reaction product is found in the extracellular space (e.g. *thick arrows*). Fine granulated reaction product occurs in the Golgi saccules (Gs) and vesicles (v), as well as in some endoplasmic reticulum cisternae (er). **B** In the neuropil, reaction product is strong along plasma membranes. Some interneuron dendrites (id), axon terminals (t) and glial processes (g) show very strong deposit intracellularly. Axon initial segments (ais) contain enzyme reaction product in smooth surfaced vesicles and tubules (see *framed area* in Fig. 6). *Scale bars* *A* 0.5 μm, *B* 1 μm

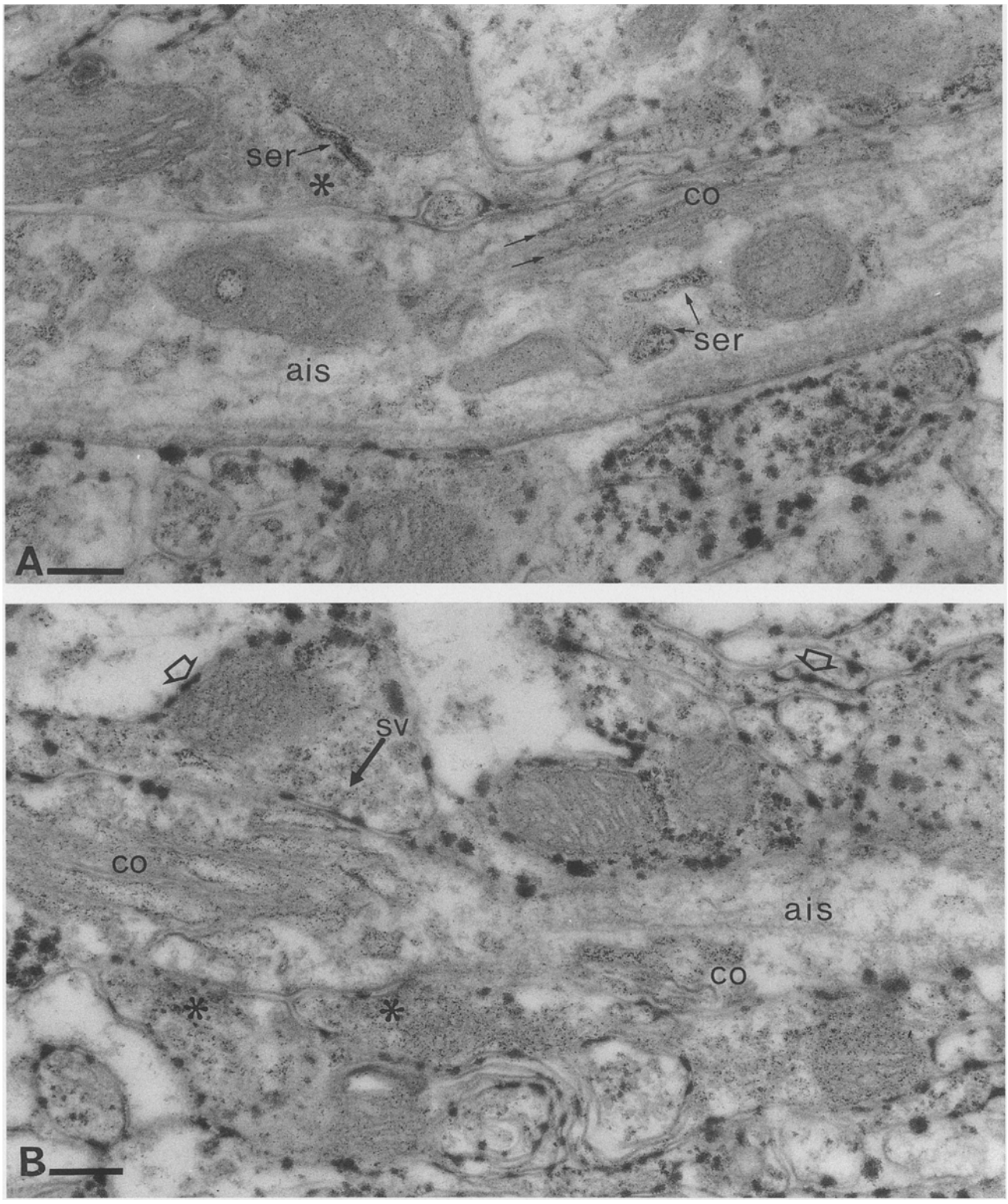

Fig. 6A Fine granulated reaction product is seen in smooth endoplasmic reticulum tubules (ser) and also in the cisternae of a cisternal organelle (*arrows*, co). This axon initial segment (ais) is shown also in Fig. 5. A tubule (ser) is labelled in an axo-axonic cell terminal (*asterisk*). **B** Cisternal organelles (co) contain fine grains as do synaptic vesicles (sv). The cisternal organelle to the *left* is cut tangentially and is near synaptic junctions (*asterisks*). Ca^{2+}-ATPase enzyme activity associated with the plasma membranes results in a coarser reaction end-product (e.g. *open arrows*). *Scale bars* 0.2 μm

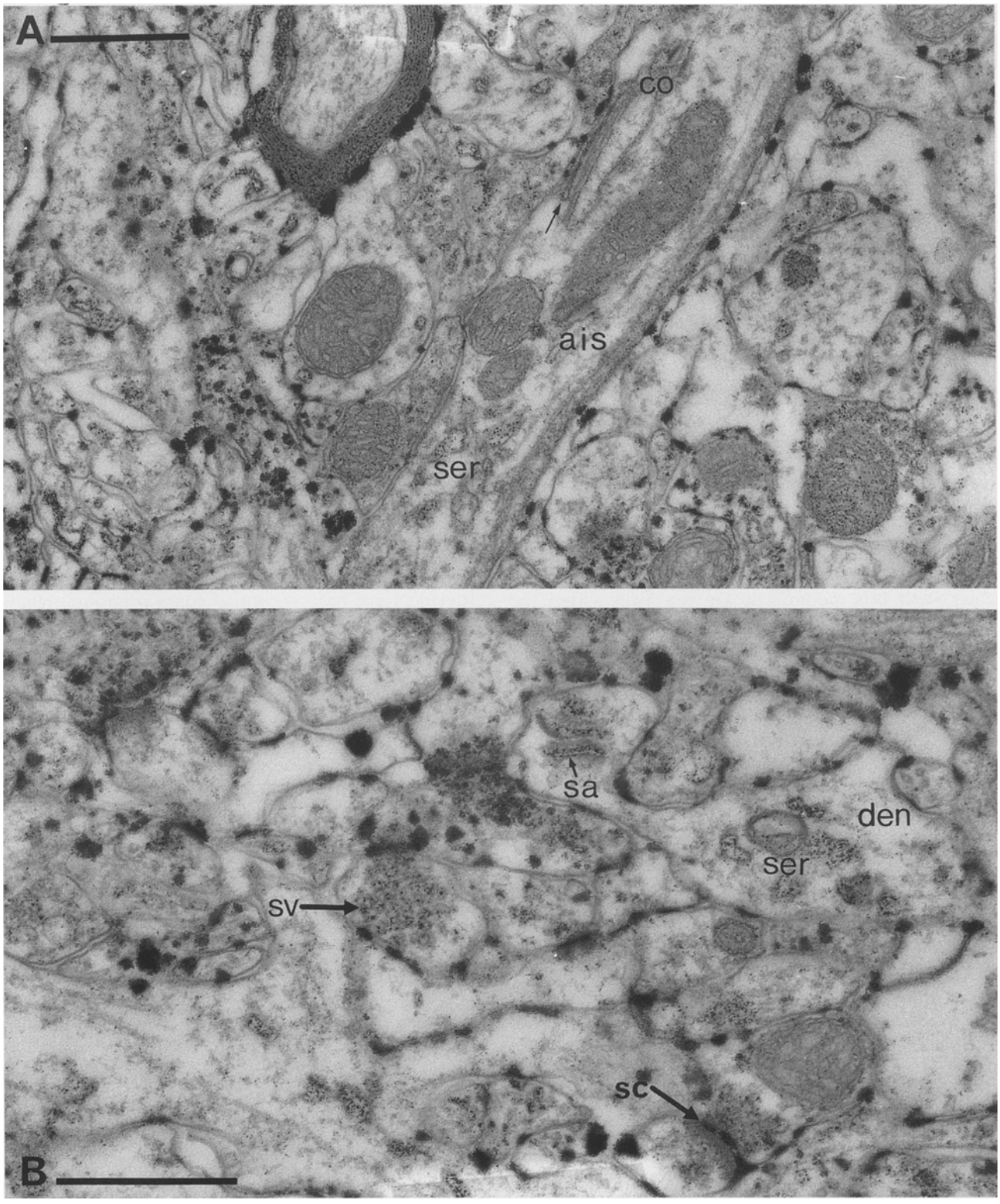

Fig. 7A Axon initial segment (ais) with a cisternal organelle (co) showing enzyme activity only in the outer cisterna (*arrow*). The smooth endoplasmic reticulum is also stained (ser). **B** Ca^{2+}-ATPase enzyme cytochemical reaction in the neuropil of stratum pyramidale. Reaction product in the spine apparatus (sa), synaptic vesicles (sv) and dendritic (den) smooth endoplasmic reticulum (ser) is similar to that in the axonal cisternal organelles. Plasma membrane associated reaction products e.g. in a synaptic cleft (sc) is more dense and deposited in larger clumps. *Scale bars* 0.5 µm

Similarities and differences between immunocytochemical and enzyme cytochemical detection of Ca^{2+}-ATPase

Cisternal organelles, positive for Ca^{2+}-ATPase, were found with both methods. Certain types of interneurones also showed very strong labelling with antibodies, as well as with the enzyme reaction. However, the immunoreacted material was uneven at any given depth of the thick sections, which were recut for electron microscopy. Antibodies do not penetrate evenly into tissue and at the same depth both immunopositive and immunonegative cisternal organelles could be found. The lack of immunoreactivity could reflect the absence of Ca^{2+}-ATPase, but since using the pre-embedding method apparent negative reactions are encountered more frequently in the depth of the sections with antibodies to tissue constituents (e.g. GABA), which are known to be present in certain elements throughout the tissue, a more likely explanation is that immunonegative cisternal organelles were not reached by antibodies. Using the enzyme cytochemical method, there was also an uneven penetration of reagents; the surface was overreacted with heavy deposit in many processes, whereas at deeper levels there was no reaction product. Nevertheless, with this method, at a given level within the thick section, more cisternal organelles were positive for Ca^{2+}-ATPase reaction than with the immunocytochemical method. The same unevenness applies to other organelles and membranes that were positive for Ca^{2+}-ATPase.

A major difference between the two methods was in the frequency of labelling throughout the tissue, the immunocytochemical reaction giving much sparser labelling. Furthermore, no clear immunolabelling of the plasma membrane was detected, but dense reaction product delineated plasma membranes following Ca^{2+}-ATPase enzyme reaction. Immunolabelling was very rare in synaptic terminals, but most synaptic vesicles usually showed fine granular Ca^{2+}-ATPase enzyme reaction. These and other differences are probably due to the differences in the amino acid sequence of different Ca^{2+}-ATPase proteins, rendering some of them unrecognisable by our antibodies, whereas the enzyme reaction would reveal most if not all Ca^{2+}-ATPase enzyme activity.

Discussion

Calcium storing organelles in neurones

The results demonstrate that the cisternal organelle membrane contains one element of calcium sequestration, a Ca^{2+} pump, which is probably of the vacuolar or SERCA2b type ATPase (Grover and Khan 1992; Plessers et al. 1991). In this respect, the cisternal organelle is similar to the endoplasmic reticulum in the somata and dendrites and to the spine apparatus in dendritic spines (Takei et al. 1992). The latter is thought to be involved in sequestering and releasing calcium in relation to synaptic input (Andrews et al. 1988; Mignery et al. 1989; Satoh et al. 1990). Cisternal organelles in the axon initial segment are a highly specialised derivative of the smooth surfaced, endoplasmic reticulum, which is generally accepted as a Ca^{2+} storing and releasing cell compartment (Berridge and Irvine 1989; Ross et al. 1989; Rossier and Putney 1991; Satoh et al. 1990). In addition, mitochondria, the rough endoplasmic reticulum and so called 'calciosomes', derived from the endoplasmic reticulum, may also function as Ca^{2+} regulating subcellular components in nerve cells (Hashimoto et al. 1988; Takei et al. 1992; Treves et al. 1990; Volpe et al. 1988; see review, Rossier and Putney 1991).

The special feature of the cisternal organelle is that it is closely associated with the plasma membrane and it is connected to it through bridges as demonstrated in the present study. The close association with the plasma membrane is reminiscent of a similar association of the so-called sub-surface cisternae in the somata and dendrites of various neurones (Henkart et al. 1976; Rosenbluth 1962). However, subsurface cisternae rarely form stacks and, when they do, no filamentous electron-dense bands are present between the cisternae. Subsurface cisternae have been shown to contain immunoreactive material resembling the gap junction proteins, connexins, and it has been suggested that these channels play a role in the entry and release of Ca^{2+} (Yamamoto et al. 1990; 1991). Interestingly, a population of nerve terminals – the so called C terminals – on spinal motoneurones is always associated with subsurface cisternae, similar to the axo-axonic cell terminals which are associated with cisternal organelles.

Cisternal organelles, sometimes called lamellar bodies, have also been demonstrated in dendrites of thalamo-recipient cortical spiny stellate cells in the monkey visual cortex (Freund et al. 1989), and in other cortical neurones (Somogyi and Cowey 1981). However, in dendrites, the organelle does not seem to be in close contact with the plasma membrane, resembling in this respect the spine apparatus.

Calcium release from intracellular stores

As in other parts of the neurone, calcium may enter the axon initial segment upon depolarisation through voltage-gated, calcium channels activated by the generation of action potentials. This calcium is extruded by the plasma membrane calcium pump and/or is taken up by intracellular organelles. Calcium uptake not only helps to keep the cytoplasmic calcium concentration low, but also provides a pool of calcium for internal signalling (see review, Henzi and MacDermott 1992). The selective enrichment of the axon initial segment of cortical pyramidal cells in cisternal organelles suggests that the accumulated calcium serves a potentially releasable pool. There are at least three possible calcium

release mechanisms that might operate in the initial segment:

1. Activation of phospholipase C, resulting either from the entry of calcium through voltage-gated Ca^{2+} channels (Audigier et al. 1988; Linden and Routtenberg 1989), or from the activation of receptors by a neuroactive peptide co-released with GABA from axo-axonic cell terminals (Lewis and Lund 1990) could lead to an increase in inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (for review, see Berridge 1993; Tsunoda, 1993). The former, together with calcium, could activate IP3 receptors in the cisternal membrane resulting in calcium release. Inositol phosphate activated calcium channels have been demonstrated in the endomembranes of many neurones, including hippocampal pyramidal cells (Maeda et al. 1989; Mignery et al. 1989; Ross et al. 1989; Satoh et al. 1990; Sharp et al. 1993; Theibert et al. 1987), therefore, this hypothesis can be tested by establishing whether IP3 receptors are present in cisternal organelles.
2. Entry of calcium into the axon could directly activate cardiac type ryanodine receptors in neurones (Kuwajima et al. 1992; Lai et al. 1992) and lead to calcium-evoked calcium release from cisternal organelles. Ryanodine binding (Padua et al. 1992) and immunoreactive ryanodine receptors have been demonstrated in hippocampal pyramidal cells (Nakanishi et al. 1992), but the resolution is not yet high enough to see their presence in axon initial segments. Ryanodine-receptor-mediated calcium release is also regulated by cyclic ADP ribose (Galione 1992), and this mechanism could also operate in hippocampal pyramidal cells, provided the signal that leads to a rise in cyclic ADP ribose concentration was present.

The problem with the two above hypotheses is that they do not explain the close association of the cisternal organelle with the plasma membrane. Both IP3 and ryanodine-receptor-mediated calcium release operate throughout the cells, not only along the plasma membrane.
3. A calcium release mechanism that requires close association of the storage compartment in the cell with the plasma membrane has been studied in detail in skeletal muscle. The muscle type of ryanodine receptor, calcium release channel is present in the sarcoplasmic reticulum membrane at the triad structure. The large cytoplasmic end-foot of the receptor/channel molecule forms a bridge to the T-tubules of the plasma membrane (Inui et al. 1987; Saito et al. 1988; Takeshima et al. 1989). The end-foot is thought to be associated with a dihydropyridine sensitive calcium channel, which acts as a voltage sensor detecting the depolarization of the plasma membrane (for review, see McPherson and Campbell 1993). Conformational change mediated by the end-foot has been suggested to act as the signal to evoke the release of calcium from the sarcoplasmic reticulum.

The skeletal muscle type ryanodine receptor is expressed in the brain, particularly in the cerebellum (Kuwajima et al. 1992). In the hippocampus, antibodies did not reveal skeletal muscle type ryanodine receptors in immunoblots (Kuwajima et al. 1992). However, if the protein was present only in the axon initial segments, as the structural characteristics suggest, it may not be sufficient for detection by this technique. Further high resolution immunocytochemical examination is required to examine the molecular composition of cisternal organelles. On the basis of the structural features, we suggest that cisternal organelles may release calcium through a mechanism similar to that found in skeletal muscle sarcoplasmic reticulum, i.e. by direct activation of a ryanodine receptor type voltage sensor/calcium channel interface in the cisternal membrane, following action potential evoked depolarisation.

The role of the cisternal organelle in regulating principal cell excitability

The possibility that cisternal organelles release Ca^{2+} upon the generation of action potentials raises the question-what is the functional significance of the compartmentalised rise in Ca^{2+} within the initial segment of pyramidal axons? The membrane of the axon initial segment contains high density of voltage-sensitive sodium channels, which are known to be regulated by Ca^{2+}/phospholipid-dependent protein kinase (PKC) requiring Ca^{2+} for activation (Numann et al. 1991). Thus, Ca^{2+} released from cisternal organelles might contribute to the modulation of sodium channels. Although this mechanism may well operate in cortical cells, if an intrinsic calcium store was required for sodium channel modulation in the axon initial segment, cisternal organelles would be expected to be widespread in neurones of other parts of the brain. The same applies to the possibility that cisternal organelles are mainly involved in Ca^{2+} uptake to keep its level low for the normal functioning of this part of the neurone.

As far as it is known, cisternal organelles in axon initial segments are unique to cortical principal cells (i.e. pyramidal cells, spiny stellate cells, dentate granule cells), and previous systematic studies in other neurones have not reported similar structures (Conradi 1969; de Zeeuw et al. 1990; Somogyi and Hamori 1976; Westrum 1993). Therefore, it is reasonable to suggest that their presence in the AIS of cortical principal cells is related to the unique source of GABAergic input to the axon from axo-axonic cells (Somogyi 1977; Somogyi et al. 1983a; 1985). Using paired intracellular recording of identified neurones, Buhl et al. (1993, 1994a) recently demonstrated that axo-axonic cells produce fast IPSPs in hippocampal principal cells and this response is blocked by the specific $GABA_A$ receptor antagonist bicuculline. The $GABA_A$ receptor complex is known to be regulated through phosphorylation by both protein kinase A, activated by cyclic AMP, and PKC, activated by diacylglycerol and Ca^{2+} (Leidenheimer et al. 1991; Raymond et al. 1993). An increase in intra-axonal Ca^{2+}

released from cisternal organelles could therefore modulate $GABA_A$ receptor function, as suggested for many different cells (see below) using diverse sources of calcium.

Increased intracellular calcium has generally been shown to down-regulate $GABA_A$ receptors in many cells (Inoue et al. 1986; Leidenheimer et al. 1991; Stelzer 1992; Whiting et al. 1990) including hippocampal pyramidal neurones (Pitler and Alger 1992). If this was the case also in the axon initial segment, repetitive firing of principal cells and the consequent increase in Ca^{2+} would lead to a down-regulation of axo-axonic cell synaptic efficacy and enhanced responsiveness of principal cells. Increased responsiveness might be adaptive once a neurone reaches threshold of firing upon receiving strong and consistent stimuli. However, the same mechanism might also lead to excessive firing due to a weakening of inhibitory control at the action potential generation site (Stuart and Sakmann 1994). A general reduction of inhibition in the cortical network leads to non-adaptive epileptic states.

In contrast to the widely observed down-regulation of $GABA_A$ receptor-mediated responses, an increase in intracellular Ca^{2+} through voltage gated calcium channels has been shown to enhance $GABA_A$-receptor-mediated chloride currents in cerebellar Purkinje cells (Llano et al. 1991). In the axon initial segment, $GABA_A$ receptors may be regulated in a similar manner by Ca^{2+}; thus the release of calcium from cisternal organelles could strengthen inhibition by axo-axonic cells. This mechanism would tend to reduce the probability of firing in proportion to the firing rate of the principal cell, and might provide a fine tuning for negative feedback on cortical cells exerted by axo-axonic cells. The recognition that the axo-axonal GABAergic synapses probably have a Ca^{2+}-mediated regulatory mechanism different from other GABAergic synapses on principal cells, which are not associated with cisternal organelles, is testable experimentally, by studying the effect of intracellular calcium levels on the inhibitory signals produced by identified types of presynaptic neurones (Buhl et al. 1994a, b).

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