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RETINAL HORIZONTAL CELLS: OLD CELLS, OLD EXPERIMENTS, NEW RESULTS

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INTRODUCTION

The first intracellular recording of the electrical activity of retinal horizontal cells was published in 1953 by Gunnar Svaetichin (56). Since then the electrophysiological study of horizontal cell responses, for a long time referred to as S-potentials in honour of the discoverer, has provided important information on the mechanisms underlying the elaboration of visual information in the outer retina and, more in general, on the processes of cell interaction in the central nervous system.

Horizontal cells were the first vertebrate neurons recorded intracellularly in the supraspinal part of the central nervous system, and were the first cells in which neural information appeared to be encoded by graded changes of membrane potential in the absence of classical action potentials. In many occasions the study of the responses of these neurons has revealed properties considerably different with respect to the behaviour of more conventional nerve cells. Eventually, however, what seemed to be a somewhat bizarre characteristic of an atypical retinal neuron turned out to be an important aspect of the mechanisms whereby local circuits in the central nervous system process neural signals (39).

Among the notions emerged from more than four decades of horizontal cell studies is the idea that a neuron may not necessarily behave as a functional unity receiving the input at the soma-dendritic region and emitting the functional output via the axon terminal according to the classical principles of neuron doctrine. Horizontal cells may receive input at both dendritic and axon terminal regions and may influence other retinal neurons through output synapses localised in any of the two regions, sometimes via local circuits which do not necessarily involve the cell as a whole (12, 39, 42).

Another important aspect of the apparently atypical behaviour of horizontal cells concerns the electrical coupling existing among adjacent cells. This coupling, which is due to the presence of numerous "gap-junctions" in the contact area of neighbouring cells (49, 61, 63), underlies the large receptive field properties of horizontal cell light responses. As a consequence of coupling, horizontal cells can play a central role in the long-distance lateral interactions among visual channels at the first synaptic stage of the retina. As a matter of fact horizontal cells may behave, from the electrical point of view, more like syncytia than as individual cells since the electrical signal spreads easily from one cell to another through the gap junction channels.

An important achievement in this field was the demonstration that the conductance of horizontal cell electrical junctions could be modulated by neurotransmitters (43, 44, 57). In particular the application of dopamine, or of agents capable of increasing the release of endogenous dopamine from the retina, reduced gap junction permeability in horizontal cells. These results represented the first demonstration of a neurotransmitter-induced control of gap junction properties in the nervous system, and brought electrical synapses, up to then considered as rigid devices of intercellular communication, in the realm of "modifiable" synapses. Subsequent investigations extended this observation to other gap junctions in both retina and in other regions of the nervous system as well, again confirming the idea that horizontal cell studies could provide important insights to the functioning of other, apparently more typical, neurons (22, 38).

In the sixties the mechanism whereby the electrical responses of horizontal cells are produced was the subject of considerable debate. In general, horizontal cells respond to light with graded hyperpolarisations, whose amplitude increases when light intensity increases. In 1965 Tomita and coworkers showed that also photoreceptors respond to the light with graded hyperpolarisations (58). A simple hypothesis to account for horizontal cell responses was that photoreceptor hyperpolarisation was transmitted to horizontal cells through electrical synapses. However, although the morphology of photoreceptor synapses did not strictly conform to that of typical chemical synapses, there was no evidence for electrical junctions between photoreceptors and horizontal cells, and a chemical transmission seemed thus more likely. Accordingly, two different possibilities could be considered: *a*) photoreceptor transmitter had a hyperpolarising action on horizontal cells and its release increased following light stimulation, or, *b*) photoreceptor transmitter had a depolarising action on horizontal cells, its release being high in the darkness thus keeping horizontal cell depolarised, and low in the presence of light, thus leading to the light-induced membrane hyperpolarisation. It may seem now difficult to conceive the possibility that a chemical transmitter is released at higher rate when the presynaptic membrane is relatively hyperpolarised (hypothesis a), since we know that neurotransmitter release is brought about by the influx of Ca^{2+} in presynaptic membrane through voltage-dependent channels opened by membrane depolarisation. However, the Ca^{2+} hypothesis of synaptic transmission was being formulated just in that period (27), and its general applicability to all chemical synapses had yet to be established.

The first clear evidence that photoreceptor transmitter had a depolarising (excitatory) action on horizontal cells was provided by Trifonov (59) in a study based on the application of short extracellular current pulses between two large electrodes situated at the vitreous and scleral side of the retina (transretinal radial currents). When the current was such as to depolarise photoreceptor synaptic terminals (positive electrode in the sclera), a large depolarising response was observed in horizontal cells. The amplitude of this response changed according to the light stimulation of the retina, and in particular it increased in the presence of moderately bright backgrounds. No comparable response was induced by currents of opposite polarity. Trifonov interpreted the depolarising response elicited in hori-

zontal cell by sclero-positive currents as a response to an increased release of a de*polarising* transmitter from photoreceptor terminals, and concluded that an excitatory transmitter was released from photoreceptor terminals at high rate in the darkness (hypothesis *b* above).

The correctness of Trifonov's conclusion was confirmed some years later by the results of a series of experiments in which the release of the synaptic transmitter was blocked by reducing Ca^{2+} concentration in the perfusing medium, by increasing Mg^{2+}, or by adding Co^{2+} ions (9, 14, 26). All these experimental manipulations led to a decrease of horizontal cell response accompanied by a *hyperpolarising* shift of the dark potential, an effect easily accounted for by the hypothesis of the depolarising transmitter. It appeared thus confirmed, in keeping with Trifonov's view, that the true resting potential of horizontal cells corresponded more to the potential observed in the presence of an intense light (when photoreceptor transmitter should be released at a low rate), than in the darkness. These experiments were later confirmed in other preparations, and, since then, Co^{2+} ions, which block synaptic transmission at relatively low concentrations, have become a common experimental tool in the study of retinal function. Furthermore, with the time the evidence has been accumulating to indicate that glutamate is the photoreceptor transmitter, and recent studies have confirmed earlier suggestions based on the observation that glutamate has a depolarising action on horizontal cell membrane (3, 8, 11, 55)

However, in the eighties several "discordant" results appeared, casting doubt on the role of Ca^{2+} in synaptic transmitter release from photoreceptors, and on the applicability of the classical scheme of chemical synaptic transmission at this level. In the amphibian retina, prolonged application of a medium containing Co^{2+} and lacking Ca^{2+}, produced only a partial block of horizontal (and bipolar cell) responses (54). In fish, the blocking effect of Co^{2+} and Ni^{2+}, also applied in the absence of Ca^{2+}, was transient, and normal horizontal cell responses reappeared with prolonged perfusion (60). In goldfish, moreover, it was reported that synaptic transmission from cones to horizontal cells was inhibited by the millimolar concentrations of Ca^{2+} normally used in the perfusing saline, and was increased by lowering Ca^{2+} in the perfusing medium to 50-100 μM (51). Finally, in turtle retina, if Mg^{2+} was removed from the perfusing medium together with Ca^{2+}, the block of horizontal cell responses was associated with a depolarising shift of the membrane potential, an effect difficult to reconcile with a block of the release of an excitatory photoreceptor transmitter (37).

On the basis of the scarce synaptic blocking efficiency of low-Ca^{2+} media containing Co^{2+}, Schwartz proposed that a significant component of transmitter release from photoreceptor occurred through a Ca^{2+}-independent process, and that it probably involved the operation of a membrane carrier according to a mechanism originally proposed by Raiteri and Levi (45).

Unexpected results also came out from experiments based on the application of transretinal currents, the same technique used by Trifonov in his classical study. Byzov (7) found that the horizontal cell block induced by Co^{2+} ions could be relieved by steady application of transretinal currents capable of depolarising

photoreceptor terminals. No comparable voltage-dependent "unblock" of Co^{2+} action had been reported for other chemical synapses, and thus this result also cast doubts on the notion that transmission from cones to horizontal cells involves a classical chemical synapse.

We were led to reinvestigate the problem of the synaptic transmission starting from experiments designated to clarify the mechanism underlying the effect of transretinal currents on the Co^{2+}-induced block (40, 41, 52). It turned out that a same explanation could account for this effect and for the apparent relative Ca^{2+}-independence of synaptic transmitter release from photoreceptors. In fact the results of our experiments may have rather general implications and could help to consider in a different light the so-called "Ca^{2+}-independent release of neurotransmitter", a notion which has gained a considerable importance in synaptic physiology in last two decades. We were surprised in realising that by largely repeating experiments made more than twenty years ago a new vista could emerge on the possible effects of divalent cations on synaptic transmission in the retina and in other regions of the nervous system. Again retina investigations appear to be a source of important information on the functional organization of the nervous system, as recognised many years ago by Cajal, who expressed his devotion to retinal studies with words full of emotion "*The retina has been always generous with me...the retina, the oldest and most persistent of my laboratory love* (48)".

METHODS

The experiments were carried out in the eyecup preparation of the turtle, *Pseudemys scripta elegans*, continuously superfused with a Ringer solution of the following composition (in mM): NaCl, 110; KCl, 2.6; $NaHCO_3$, 22; $MgCl_2$, 2; $CaCl_2$, 2; D-glucose, 10, bubbled continuously with a mixture of 95% O_2, 5% CO_2 to bring the pH to 7.4 (see ref. (43) for experimental details). They consisted in the intracellular recording of the light responses induced in horizontal cells (and other retinal neurons) by 5 mm-diameter white-light spots of variable intensity and duration. All the horizontal cell responses illustrated in this article were obtained from the axon-terminal of the H1- or luminosity-type horizontal cell (31). Transretinal currents were applied between two large silver-chloride electrodes positioned at the scleral and at vitreous side of the retina, respectively (see ref. (7) for experimental details).

RESULTS

Figure 1 illustrates the results of an experiments in which the effect of sclero-positive transretinal current were investigated on the block of the horizontal cell light responses induced by Co^{2+} ions. The tracings on the left were obtained in control conditions (*a*), and 5 and 9 minutes respectively after the application of 4 μM Co^{2+} (*b* and *c*). As previously reported (9) Co^{2+} application resulted in an almost complete block of the horizontal cell response and in a hyperpolarising shift of the dark potential, an effect consistent with Trifonov's hypothesis that horizon-

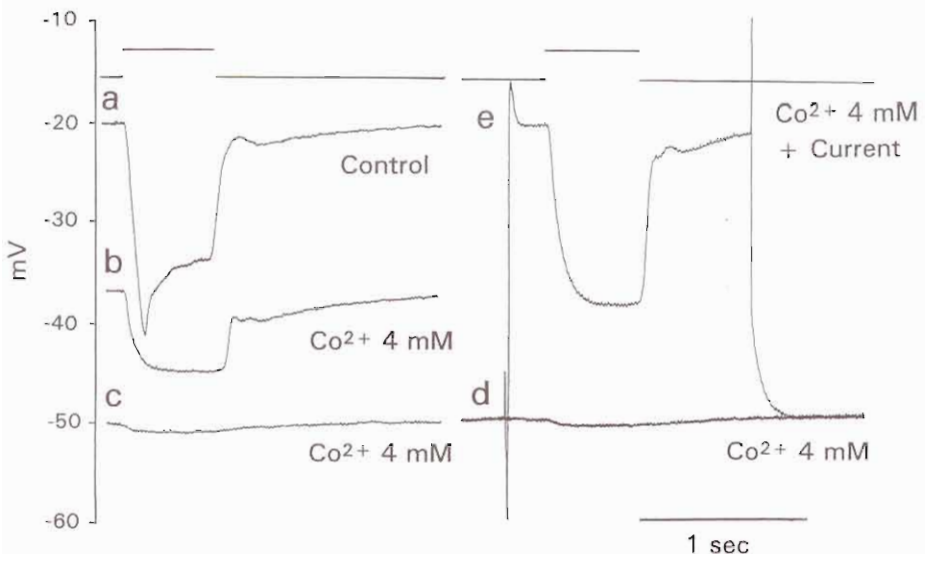

Fig. 1. - *Effect of transretinal current on the block of horizontal cell light responses induced by the application Co^{2+} ions.*

The left panel illustrates the light responses elicited in a horizontal cell by retinal illumination with a 410-ms white light step (monitored above the top recording) in control conditions (*a*), and 7 (*b*) or 10 minutes (*c*) after the application of a saline containing 4 μM Co^{2+}. The recordings at the right were taken soon after recording (*c*) and illustrate the responses in the absence (*d*), or during the passage of a 30 μA extracellular current (*e*) applied from the sclera (positive electrode) to the vitreous side (as indicated). The current resulted in a membrane depolarization and an almost complete recovery of light response amplitude.

tal cell light responses result from a reduction of the flow of an excitatory transmitter from photoreceptor terminals. In the presence of Co^{2+}, electrical stimulation of the retina with a sclero-positive current resulted in an almost complete recovery of the horizontal cell response (tracing *d* and *e* on the right). A similar effect was observed in all horizontal cells in which the effects of Co^{2+} (1-5 μM) were investigated. It was consistently observed, however, that progressively larger current intensities were necessary to counteract the response block if Co^{2+} application was prolonged, particularly at high Co^{2+} concentrations, and eventually only a partial recovery was possible with the highest current intensity available.

These results were in agreement with the previous observations of Byzov mentioned above. In order to investigate the mechanism of transretinal current effects we studied next the action of the same currents on the block induced by other divalent cations. We found that sclero-positive currents could also counteract horizontal cell-response block induced by Zn^{2+} (0.2-1 μM) and Ni^{2+} (0.5-2 μM). In fact transretinal currents were more powerful in relieving Zn^{2+} and Ni^{2+}-induced block than the block brought about by Co^{2+}. Similar to the Co^{2+} experiments, also with Zn^{2+} and Ni^{2+} progressively larger current intensities were necessary when the

application of the blocking ion was continued for long times, and eventually only a partial recovery could be obtained. On the other hand, transretinal currents produced a modest recovery from the block induced by Cd^{2+} (0.2-1 μM) and Mg^{2+} (15-20 μM), and could not counteract the horizontal cell-response block induced by low Ca^{2+} media (with or without 2-4 μM EGTA added). Finally, no recovery of light responses was observed when the depolarising currents were applied after blocking horizontal cell light responses with the glutamate antagonist kynurenic acid (1-2 μM), thus suggesting that the current-induced unblock was not due to a postsynaptic action of the current on the horizontal cell membrane.

The ensemble of these experiments indicated that the main effect of transretinal current was presynaptic, and possibly involved a voltage-dependent "unblock" of the effects of Zn^{2+}, Ni^{2+} and Co^{2+}. Moreover, Mg^{2+}, Cd^{2+} and Mn^{2+} seemed to differ in the mechanism of their blocking action compared to Zn^{2+}, Ni^{2+} and Co^{2+}.

The Ca^{2+} channels present in photoreceptors belong almost exclusively to the dihydropyridine-sensitive L-type channels (4, 17, 29, 32), and there is no evidence that the blocking effect exerted in L-type channels by Zn^{2+}, Ni^{2+} and Co^{2+} can be relieved by depolarisations of less than 10-20 mV amplitude, similar to those induced in photoreceptor synaptic membrane by our transretinal currents (see Ref. 7). It was thus necessary to consider a different mechanism to account for the effect of transretinal currents. A first hint came from the consideration that transretinal currents were strongly effective only in the initial phase of the blocking action of Zn^{2+}, Ni^{2+} and Co^{2+}, and that their unblocking power declined with prolonged divalent application. We speculated that the initial response block could be due to low levels of the divalents cations, before the concentration in the synaptic space equilibrated with that of the perfusing saline, and that this effect did not involve a real block of presynaptic Ca^{2+} channels. In trying to figure out how low concentrations of Zn^{2+}, Ni^{2+} and Co^{2+} could lead to a suppression of synaptic transmission, we considered the possibility that the effect of these ions was mediated by a reduction of Ca^{2+} influx due to an action on the fixed negative charges present on the membrane surface according to the predictions of the surface-charge theory. This possibility appeared particularly likely because Zn^{2+}, Ni^{2+} and Co^{2+} are the divalent cations most effective in neutralising membrane surface charges, while Mg^{2+} has a weak action at this level (16,25,35). Before referring the results of our further experiments, which were fully consistent with this possibility, we will give now a rapid outline of the surface charge theory, and we will try to explain why a neutralisation of these charges brought about by divalent cations, may lead to a block of synaptic transmission.

1. *Outline of the surface charge theory.*

The plasma membrane of all normal cells bears at its external surface an excess of fixed negative charges, mostly due to acidic phospholipids of the lipid bilayer, but in part also to other membrane components (proteins, sialic acid, gangliosides (see Refs. 33 and 24). The presence of these fixed charges modifies the local environment in the aqueous phase surrounding the membrane. Fixed charges

polarise the surrounding medium and establish a surface potential which decays in a quasi-exponential way toward the aqueous medium with a space constant of about 1 nm in physiological conditions. Due to its rapid decay this surface potential cannot be measured with conventional microelectrode techniques. It can be, however, evaluated with indirect methods, such as electrophoresis of entire cells or membrane fractions, or with fluorescence studies using probes which partition between membrane surface and extracellular bulk solution according to the value of the surface potential (13, 15, 36). Surface potential can be of quite large amplitude (-100 mV or more in some particular conditions), and thus can completely overcome the electrical field established across the membrane by electrochemical gradients. Surface potential can change in physiological conditions due to changes in the ionic composition of the extracellular medium. It is in the mutual interactions which exist between the negative fixed charges on the membrane surface and the ions in the surrounding medium that resides a crucial aspect of surface charge relevance to membrane physiology (19, 24, 30, 33).

A first aspect of these interactions concerns the influence of ions on the surface potential. In particular, positive ions can neutralise fixed charges by electrostatic screening. Since this effect depends on the ionic charge, divalent cations are more effective than monovalents at this level as observed experimentally. Moreover, different divalent cations show a different effectiveness in their neutralising action on surface charges, with Zn^{2+}, Ni^{2+} and Co^{2+} being the most powerful, Cd^{2+}, Mn^{2+} and Ca^{2+} having an intermediate potency, and Mg^{2+}, Ba^{2+} and Sr^{2+} being the less effective (16, 25, 5). This difference, not accounted for by simple electrostatic considerations, is supposed to reflect a true binding of cations to the membrane surface, a binding which varies according to the ionic species (34). The most important divalent cation in influencing surface potential in physiological conditions is Ca^{2+}. When extracellular Ca^{2+} concentration rises, the surface potential decreases, and the opposite occurs when Ca^{2+} concentration goes down. A reduction of surface potential can also be obtained by introducing into the extracellular medium other divalent cations, and, in particular, Zn^{2+}, Ni^{2+} and Co^{2+} which, as mentioned above, are considerably more effective than Ca^{2+} in neutralising surface charges. For what concerns the electrical field acting on the voltage sensors located in the thickness of the membrane, the effects of an increase of Ca^{2+}, or of the addition of exogenous divalents, are indistinguishable from those of a membrane hyperpolarisation since a reduction of external negativity is in this respect equivalent to an increase of internal negativity. Therefore, the opening of voltage-dependent ionic channels which are normally activated by membrane depolarisation (classical Na^+ channels, many Ca^{2+} channels, some K^+ channels) will require stronger membrane depolarisations to be activated in these conditions. This "positive shift" of activation curve in high divalent concentrations has been in fact observed experimentally for all channels investigated.

On the other hand, when the concentration of divalent cations in the extracellular medium is decreased, the membrane will experience a reduced electrical field across its thickness, and this situation corresponds to that normally induced by a

depolarising stimulus. As a consequence, the voltage-activated ionic channels which are gated by membrane depolarisation would be opened by smaller membrane depolarisations in the presence of a reduced divalent concentration. This "negative shift" of the activation curve has also been reported for all voltage-dependent ionic channels investigated when extracellular Ca^{2+} concentration is decreased. It has practical consequences since it accounts for the hyperexcitability syndrome brought about by decrease of plasma Ca^{2+} levels which occurs in some pathological conditions, as for instance following a reduction of parathyroid hormone secretion (*"tetania parathyreopriva*).

The second important aspect of the interactions between fixed membrane charges and ions in the extracellular medium concerns the influence exerted on ions by fixed charges. Through attractive or repulsive electrostatic forces, fixed charges lead to an increase of positive ion concentration, and to a decrease of negative ion concentration in the neighbouring of the membrane. This influence can be quite strong so that membrane ionic concentrations can be by several orders of magnitudes different as compared to the concentrations in the bulk extracellular medium. This occurs particularly for divalent cations, since ion distribution depends on the ionic valence (and on surface potential) according to an exponential, Boltzmann, function. Moreover, since surface potential increases when concentration of divalent cations decreases, the difference between bulk and membrane concentration may become particularly large (even more than 10,000 fold) at low divalent levels (see Refs. 30, 33,62). As a consequence, when extracellular divalent concentration becomes very small, the membrane concentration of these ions becomes practically insensitive to changes of the bulk concentration.

The influence of surface charges on local ionic concentrations in the proximity of the membrane, and in particular the higher local concentrations of positive ions, may have important functional consequences for current flow across ionic channels. Ion permeation through a membrane channel does not depend exclusively on the electrochemical gradient of the permeant ion between the intracellular and extracellular compartments, which is largely independent on the presence of surface charges. It depends also in an important way on the local concentration of the permeant ion at the mouth of the channel, and this can be profoundly affected by surface potential. In particular, through the influence of surface charges, a cationic channel may have a much higher permeability than it could be supposed by considerations of the bulk concentration of the ion (19,30). For the reasons exposed above this effect is particularly important for divalent cations, and, moreover, it becomes prominent at low bulk concentrations.

Finally, the presence of a high concentration of positive ions near the membrane surface may interfere with the attempt to modify experimentally cation concentrations in the extracellular space. Due to the strong action of surface charges on divalent cations, this effect will also be particularly important for divalent cations, and, as we shall see, it will acquire a particular relevance during experimental manipulations aimed at reducing extracellular divalent concentrations to very low levels.

2. *CA^{2+} currents and the effect of divalent cations.*

As discussed above, a change of divalent ion concentration can affect gating and permeation in all voltage gated ionic channels. The influence of divalent cations on ionic channels has a particular connotation, however, when Ca^{2+} channels are concerned. This happens because most divalent cations have on Ca^{2+} channels other effects beside those consequent to surface charge modifications. Some ions, notably Ca^{2+}, but also Ba^{2+} and Sr^{2+}, permeate Ca^{2+} channels, while many others, such as for instance Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mg^{2+} have antagonistic action and may block directly Ca^{2+} channels by competing with Ca^{2+} (20,21). Moreover, as we have already mentioned, various divalent cations have a different efficacy in neutralising surface charges. The actual modification of Ca^{2+} influx brought about by a divalent cation would depend on the interplay of the different actions, and would be different for the different cations. It would also depend in an important way on the membrane potential. This happens because of the shift of activation curve along the voltage axis induced by divalent cations through the surface charge effect. In general, the maximal current trough Ca^{2+} channels (which is normally elicited by relatively large depolarising stimuli), would be reduced by cations with antagonistic effect on Ca^{2+} channels, and would be increased by cations capable of permeating these channels. However, due to the depolarising shift of the activation curve, an increase of the concentration of all divalents will tend to reduce the current across Ca^{2+} channels at relatively hyperpolarised potentials, near the foot of the activation curve (and a decrease of divalent concentration would have the opposite effect). An interesting, and somewhat paradoxical, possible consequence of surface charge effects is that at relatively hyperpolarised potentials the influx of Ca^{2+} could be *increased* by a *decrease* of Ca^{2+} concentration, and, viceversa, it could be decreased by a Ca^{2+} concentration increase.

Due to surface charge effects, the application of divalent cations could reduce the influx of Ca^{2+}, and, by this way, can suppress synaptic transmission, even in the absence of a true channel block. This would happen more likely for those ions, like Zn^{2+}, Ni^{2+} and Co^{2+}, which are very powerful in neutralising surface charges. For the consideration exposed above, through a surface charge effect also an increase of Ca^{2+} ions could result in a reduction of Ca^{2+} influx. By this way a Ca^{2+} increase could thus result in a reduction of synaptic transmitter release, particularly of the transmitter release observed at relatively hyperpolarised potential (basal release for instance). On the other hand, a reduction of the concentration of all divalent cations, and in some conditions also a *reduction of Ca^{2+} concentration* could result in *an increase of transmitter release.*

3. *A "surface charge" hypothesis on the effect of divalent cations on synaptic transmission in the retina.*

On the basis of the surface charge theory mentioned above it is possible to formulate a simple hypothesis to account for the "unblocking" effect of transretinal currents on horizontal cell response block brought about by Zn^{2+}, Ni^{2+} and Co^{2+}. As we have repeatedly mentioned, these ions are the most powerful in neutralising the

surface charges present on cell plasma membrane. It can be supposed, therefore, that they block synaptic transmission in the retina largely because they displace the activation curve of photoreceptor Ca^{2+} current toward more depolarised potentials, such as to reduce the influx of Ca^{2+} in photoreceptor synaptic terminals to levels incompatible with the release of chemical transmitter. This is a likely possibility because the resting potential of photoreceptors is about -35 mV (the "dark" potential), and photoreceptor Ca^{2+} current begins to activate at about -40 mV. As a consequence, a shift of Ca^{2+} current activation curve of about -5 mV could thus result in an almost complete block of synaptic transmitter release in the dark. Since, moreover, the physiological response of photoreceptors is a light induced hyperpolarisation, no synaptic transmission would be possible in these conditions. On the other hand, a depolarisation of photoreceptor synaptic terminals, such as that brought about by sclero-positive transretinal currents, would reposition photoreceptor membrane potential at a level where Ca^{2+} current can still be regulated by membrane potential, thereby restoring synaptic transmission.

In contrast to the effect of Zn^{2+}, Ni^{2+} and Co^{2+}, in our experiments the horizontal cell response block induced by Mg^{2+} and by Cd^{2+} could not be relieved by transretinal currents. It is probable therefore that Mg^{2+} and Cd^{2+} act mainly by a different mechanism, and likely they reduce Ca^{2+} influx into photoreceptor terminals via a true competition with Ca^{2+} ions in the Ca^{2+} channel.

There is an important operative difference between a true competitive block and an effect mediated by a surface charge-induced positive displacement of the activation curve. According to the classical prediction of competitive kinetics the blocking action of an antagonist ligand should be potentiated by a reduction of the concentration of the agonist (20). Thus a reduction of Ca^{2+} concentration should potentiate the blocking efficiency of divalent cations acting through a true competitive blocking action. On the other hand, a reduction of Ca^{2+} concentration should possibly counteract the action of the divalent cations which act mainly through a surface charge effect. This is because lowering Ca^{2+} level should result in a negative shift of the activation curve, and thus should reposition the activation curve within the physiological range of photoreceptor membrane potential.

In sum, a competitive action of an antagonist divalent cation on Ca^{2+} permeation across Ca^{2+} channels should be intensified by lowering Ca^{2+} concentration, whereas a reduction of Ca^{2+} could result in an increased Ca^{2+} influx, if the effect of the antagonist divalent is mediated mainly by a modification of the surface potential.

4. *The effects of lowering external Ca^{2+} concentration on the blocking effect of divalent cations.*

Figure 2 illustrates the results of an experiment in which the effect of low extracellular Ca^{2+} on the horizontal cell response block induced by Co^{2+} was investigated. The retina was first perfused with a solution containing 1 μM Co^{2+} and a normal concentration of Ca^{2+} (2 μM), and this resulted in a drastic reduction of response amplitude and in a hyperpolarisation of membrane potential. Afterwards the saline solution was changed to one still containing Co^{2+}, but without Ca^{2+}

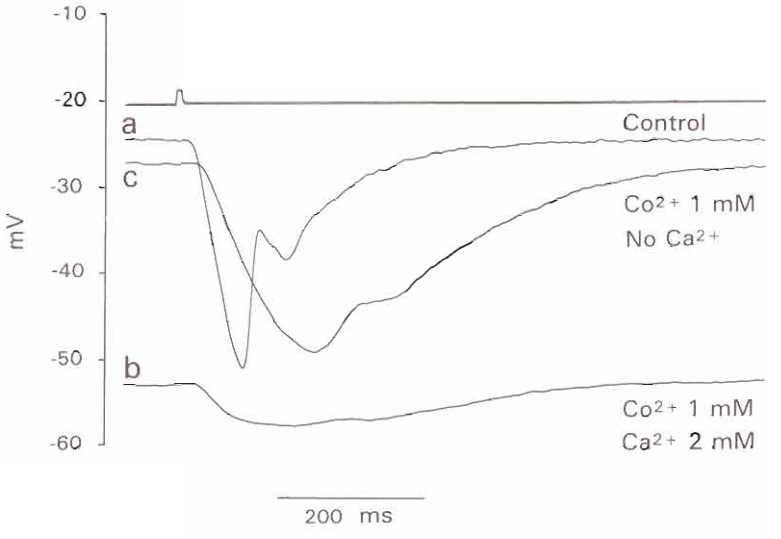

Fig. 2. - *Recovery of the Co2+-induced horizontal cell response block brought about by the application of a nominally zero Ca2+ saline.*

The light responses induced by a 10-ms light flash (monitored at the top) were recorded, respectively, in control conditions (*a*), in a saline containing 1 mM $CoCl_2$ and a normal Ca^{2+} concentration (applied for 22 minutes, tracing *b*), as well as after the application of a medium still

still containing 1 mM $CoCl_2$ but without Ca^{2+} added (10 minutes after the application of the Ca^{2+}-free solution, tracing *c*), as indicated. Application of the Ca^{2+}-free medium resulted in an almost complete recovery of dark potential level and response amplitude.

added. The response recovered, and after about 10 minutes it attained an amplitude similar than in control conditions. This paradoxical low Ca^{2+}-induced recovery of the horizontal cell responses blocked by divalent antagonists was observed in other experiments in which the effect of lowering external Ca^{2+} were investigated on various concentrations of Co^{2+} (0.5-3 μM), and, moreover, on the blocking action of Zn^{2+} (0.2-0.6) and of Ni^{2+} (0.5-2 μM). In experiments in which the effects of different concentrations of Ca2+ were studied, the unblocking effect of low Ca^{2+} was already detectable at 1 μM Ca^{2+} concentration (half than the physiological concentration) and was practical maximal at 0.2 μM. A substantial unblocking effect was seen, however, only if low Ca^{2+} solutions were applied during the initial phase of the blocking action of Co^{2+}, Zn^{2+} and Ni^{2+}. If the application of the antagonist divalents was continued for long periods, the unblocking efficiency of the of low Ca^{2+} media declined. This progressive reduction of low Ca^{2+} effect is reminding of the similar loss of unblocking action of transretinal depolarising currents upon prolonged application of Zn^{2+}, Ni^{2+} and Co^{2}.

The unblocking effect of low Ca^{2+} solutions on the initial blocking effect exerted by Zn^{2+}, Ni^{2+} and Co^{2+} on horizontal cell responses strongly argues against the possibility that action of these antagonist divalents is mediated by a true channel block. As we have discussed above, a true channel block due to a competitive

action of the antagonist divalent cations should have been intensified by reducing extracellular Ca^{2+}. Since this is the type of block normally produced by high concentrations of the divalent antagonists on Ca^{2+} channels, it is likely that the initial blocking effect of Zn^{2+}, Ni^{2+} and Co^{2+} on horizontal cell responses is due to lower extracellular concentrations of these ions before their level in the proximity of photoreceptor terminals reaches the high concentration present in the perfusing saline. Since low concentrations of Zn^{2+}, Ni^{2+} and Co^{2+} may shift considerably the activation curve of Ca^{2+} channels, without exerting a strong channel blocking effect, it is then possible that the horizontal cell response suppression is mediated by this mechanism. The surface charge hypothesis accounts in a simple way for both the unblocking action of currents which depolarise presynaptic terminals (transretinal currents) and the action of low Ca^{2+} media. The first experimental manoeuvre would restore synaptic transmission because it would displace presynaptic membrane potential at a level where Ca^{2+} current can still be modulated as a consequence of physiological light responses of photoreceptors. The second manoeuvre would reposition Ca^{2+} current activation curve within the physiological range of photoreceptor membrane potential because a reduction of Ca^{2+} concentration may contrast the surface potential modification brought about by application of exogenous divalent cations.

In contrast with the powerful efficacy in relieving the horizontal cell response block induced by Zn^{2+}, Ni^{2+} and Co^{2+}, low Ca^{2+} media did not restore horizontal cell responses blocked by Mg^{2+} or by Cd^{2+}. This insensitivity of the effects of Mg^{2+} and Cd^{2+} to the application of low Ca^{2+} media, parallells the similar insensitivity to the recovering effect of depolarising transretinal currents. As a matter of fact, the application of low Ca^{2+} resulted in an intensification of the block induced by Mg^{2+} and Cd^{2+}. From the considerations discussed above it is probable therefore that the action of these antagonist divalents is mainly due to a true Ca^{2+} channel block.

5. *The effects of low Ca^{2+} media in the absence of exogenous divalent cations.*

The unblocking action of low Ca^{2+} media on horizontal cell response in the presence of Zn^{2+}, Ni^{2+} and Co^{2+} means that low Ca^{2+} is able to increase the release of photoreceptor synaptic transmitter in the presence of these exogenous divalent cations. The question arises whether lowering Ca^{2+} may result in an increase of synaptic transmitter release even in the absence of exogenous divalents. The interest of this question is also in the fact that in the previous study of turtle retina it was found that a reduction of Ca^{2+} concentration brought about by the application of the Ca^{2+} buffer, EDTA, resulted in a hyperpolarising block of horizontal cell light response, i.e. in an effect compatible with a *reduction* of synaptic transmitter release (9). To clarify this point, and to ascertain whether the effects of low Ca^{2+} media could differ in different experimental conditions, we applied to the retina low Ca^{2+} solutions not containing exogenous divalents. We found that the final effect of these solutions differed in an important way depending on whether Mg^{2+} was present or not in the perfusing medium. Initially the application of media with no Ca^{2+}-added resulted in a depolarising shift of membrane potential and in a

reduction of response amplitude. If Mg^{2+} was absent from the perfusing medium this effect eventually developed in a full depolarising block of horizontal cell light response, i.e. with an effect compatible with a large increase of the transmitter release. In contrast, when Mg^{2+} was present in the low Ca^{2+} media, an opposite effect ensued, that is a *hyperpolarising* block of horizontal cell responses, an effect compatible with a great reduction of transmitter release. That the depolarising effect of low Ca^{2+}-low Mg^{2+} media was actually due the increase of photoreceptor transmitter release was confirmed, among others things, by the results of experiments in which the addition of the glutamate receptor antagonist kynurenic acid to these media resulted in hyperpolarisation of horizontal cell membrane. Effects similar to those induced by nominally zero Ca^{2+} solutions were also brought about by solutions containing millimolar concentrations of Ca^{2+} buffers (EGTA and EGTA), the main difference being the faster development of response and membrane potential changes. In particular, low Ca^{2+} media containing Mg^{2+}, and with EGTA or EDTA added, resulted in a relatively rapid hyperpolarising block of horizontal cell response, an effect consistent with previous studies in the same synapse (9).

Taking into account the effects of low Ca^{2+} solutions in the presence of antagonist divalents previously discussed, we can interpret these results as evidence that low Ca^{2+} media not only can restore the transmitter release blocked by some divalent cations, but they can also produce an increase of photoreceptor transmitter in the absence of exogenous divalents. The hyperpolarising block of light responses induced by low Ca^{2+} solutions containing Mg^{2+} is likely due to a true competitive block of Ca^{2+} channels brought about by the physiological concentration of Mg^{2+} when Ca^{2+} concentration becomes low.

DISCUSSION

Our interpretation can account for the effects of manipulations of divalent cation concentration on synaptic transmission from photoreceptors to horizontal cells without invoking unconventional mechanisms of transmitter release, and in particular without assuming a Ca^{2+}-independence of the transmission process. Negative surface charges are present in the membrane of all cells, and surface-charge mediated effects of divalent cations on voltage-dependent channels including Ca^{2+} channels are also common to all cells. It is thus possible that low Ca^{2+} media have similar effects on Ca^{2+} currents and on synaptic transmission in other sites of the nervous system. From previous studies it appears in fact that a reduction of Ca^{2+} concentration reduces Ca^{2+} influx at relatively depolarised potentials, but increases Ca^{2+} current at hyperpolarised potentials (near the foot of the activation curve) in other nerve cells beside retinal photoreceptors (6, 28, 53, 62, 29). So it is very probable that the release of synaptic transmitter could be increased by low Ca^{2+} also outside the retina. This should happen particularly for the release which occurs at relatively hyperpolarised potentials (the basal release, or the release

induced by stimuli which elicit relatively small depolarising shifts of the membrane potential). These are indeed the experimental conditions from which the notion of a "Ca^{2+}-independent release" has emerged (see 1, 5, 46 for review). The neurochemical and pharmacological studies which have led to the proposal of this unconventional mechanism have in fact shown that the release which persists, or is increased, in low Ca^{2+} media is, in general, the basal release or the release observed in response to chemical agents which likely evoke small amplitude depolarisations (high potassium, veratridine, excitatory aminoacids). Consistent with the hypothesis that the so-called "Ca^{2+}-independent release" in other preparations is in fact mediated by the influx of Ca^{2+} through typical Ca^{2+} channels is the observation that, as in photoreceptor synapse, this release is, in general, blocked by Cd^{2+} or by high Mg^{2+}, two divalent cations, which exert a true blocking action on Ca^{2+} channels.

In sum, according to our hypothesis, the "Ca^{2+}-independent release", in retina, and possibly in other preparations, could be explained in an economical way within the framework of the classical Ca^{2+} hypothesis of synaptic transmission, by simply taking into the due account the surface-charge mediated effects of low Ca^{2+} media.

There is, however, an important difficulty to consider at this point. In principle the surface-charge effect cannot account for the release which persists in, or is increased by, very low Ca^{2+} concentrations, so low that they cannot insure a Ca^{2+} influx sufficient to induce a transmitter release even though all Ca^{2+} channels are opened, or, in the limit, so low that they would possibly lead to an outflow of Ca^{2+} when Ca^{2+} channels open. Since, according to recent studies, release of neurotransmitter in classical synapses is activated when intracellular Ca^{2+} near the release sites reaches concentrations of 100 μM or more (2, 23, 50), our hypothesis cannot thus account for the increased release observed in experiments in which Ca^{2+} concentration in the perfusion medium is lowered to pico-nanomolar values, as normally occurs by using Ca^{2+} buffers. However, it is not correct to assume in a simple way that the divalent ion concentration in the extracellular space near synapse sites equilibrates more or less rapidly with that in the perfusing medium. Extracellular space in the nervous system is extremely thin (10-20 μm) and this already sets an important limitation to the diffusion process. Moreover, for charged molecules, and in particular for divalent ions, the problem acquires a particular connotation due to the presence of surface charges on the membranes of cells delimiting the extracellular compartment. As previously discussed, for a liquid layer of about 1 nm thickness adjacent to the membrane, the concentration of divalent cations can be much higher than in the bulk of the extracellular medium due to the surface charge influence. The difference becomes particularly large when divalent concentration is decreased due to the increased negativity of surface potential. Therefore in conditions aimed to lower extracellular Ca^{2+} to very low levels, we have to face the consequences of the "buffering action" of the external surface of cell membrane, and this can overcome the buffering power of the perfusing solution. It is thus possible that, even with prolonged application of media containing Ca^{2+}-buffers, the concentration of Ca^{2+} in the extracellular space of the nervous system

never gets so low such as to cause a synaptic transmission block via a reduced electrochemical gradient for Ca^{2+} ions. With this considerations in mind, it is possible to account within the framework of the Ca^{2+} hypothesis for the results of many studies in which transmitter release was found to persist, or to increase, following application of low Ca^{2+} media. These conditions which seem unfavourable to Ca^{2+} entry (54) may in fact promote Ca^{2+} influx.

Further studies are necessary to ascertain whether the surface charge based interpretation of low Ca^{2+} effects on synaptic transmission can account for the apparent "Ca^{2+}-independent" release mechanisms described at vary levels of the nervous system. However, on the basis of the results of our studies, the evidence for a genuine Ca^{2+}-independence of synaptic transmission cannot be based only on the effects of low Ca^{2+} media application, particularly in preparations in which the integrity of the extracellular space is preserved. It is to be expected that lowering extracellular Ca^{2+} may result, in fact, in a greater influx of Ca^{2+} through the plasma membrane of many excitable cells. A more stringent test would be, in our opinion, to apply divalent antagonists, which like Cd^{2+} and Mg^{2+}, likely exert a true blocking effect on Ca^{2+} channels. Moreover, one should be aware of the possibility that lowering Ca^{2+} in the perfusing medium does not necessarily reinforce, but, paradoxically, may actually decrease the synaptic blocking effectiveness of divalent cations like Co^{2+}, Ni^{2+} and Zn^{2+} (54, 60).

In conclusion, it may seem counter-intuitive and paradoxical that lowering Ca^{2+} could enhance the classical "Ca^{2+}-dependent" synaptic transmission and relieve the synaptic transmission block brought about by divalent cations traditionally supposed to act as Ca^{2+} channel blockers. However, from another point of view, it is also paradoxical that many workers in the field of synaptic physiology have neglected the possibility of electrostatic interactions between membrane fixed charges and ions in the extracellular medium. This has occurred in spite of the existence of a classical theory capable of accounting for these interactions, the "fixed charge theory" formulated more than 80 years ago by Gouy (18) and by Chapman (10).

As a matter of fact science is rarely the acknowledgment of simple and intuitive facts, and, in spite of their limitations, scientific theories are necessary for the progress of scientific knowledge because, as Cajal has neatly stated, a theory is "our best instrument to open a breach in the hard block of reality"(47).

SUMMARY

The study of neural interactions in the vertebrate retina carried out after the pioneering studies of Svaetichin has provided important information on the functioning of nerve circuits in the central nervous system. Recently we have investigated the effects of changes of divalent cation concentration on the synaptic transmission between cones and horizontal cells of the turtle retina. Our results seemed apparently in contrast with the classical Ca^{2+}-hypothesis of chemical synaptic

transmission. Application of low Ca^{2+} media resulted in a recovery of synaptic transmission after application of divalent cations such as Ca^{2+}, Zn^{2+} and Ni^{2+} traditionally considered as Ca^{2+} channel antagonists. Moreover, in the absence of exogenous divalent cations, low Ca^{2+} could result in an increase of transmitter release particularly if Mg^{2+} was omitted from the perfusing medium. These apparently paradoxical results can be reconciled with the postulates of the Ca^{2+}-hypothesis of synaptic transmission by taking into account the effects of divalent cations on the fixed charges present at the external surface of cell membrane. It is possible that a similar interpretation could also account for the so-called "Ca^{2+}-independent" transmission in other structures of the nervous system.

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