Resistance of Retinal Extracellular Space to Ca\textsuperscript{2+} Level Decrease: Implications for the Synaptic Effects of Divalent Cations

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Dmitriev, Andrey, Angela Pignatelli, and Marco Piccolino. Resistance of retinal extracellular space to Ca\textsuperscript{2+} level decrease: implications for the synaptic effects of divalent cations. J. Neurophysiol. 82: 283–289, 1999. Ion-sensitive microelectrodes were used to measure the variations of [Ca\textsuperscript{2+}]\textsubscript{o} induced by application of low Ca\textsuperscript{2+} media in the superfused eyecup preparation of the Pseudemys turtle. The aim of the experiments was to evaluate the possibility, suggested by previous studies, that in the deep, scleral, layers of the retina [Ca\textsuperscript{2+}]\textsubscript{o} may remain high enough to sustain chemical synaptic transmission even after prolonged application of low-Ca\textsuperscript{2+} saline. It was found that, at depths of 100–200 μm from the vitreal surface, [Ca\textsuperscript{2+}]\textsubscript{o} did not fall below 1 mM even after application for periods of 30–60 min of nominally Ca\textsuperscript{2+}-free media, and it was >0.3 mM after 30-min application of media containing EGTA and with a Ca\textsuperscript{2+} concentration of 1 mM. Previous studies in isolated salamander photoreceptors have shown that a reduction of [Ca\textsuperscript{2+}]\textsubscript{i} to 0.3–1.0 mM may result in a paradoxical increase of Ca\textsuperscript{2+} influx into synaptic terminals due to the reduced screening of negative charge on the external face of the plasma membrane. On the basis of these results, the persistence or reduced screening of negative charge on the external face of the plasma membrane. This is commonly done, for instance, to block chemical synaptic transmission (Cervetto and Piccolino 1974; Kaneko and Shimazaki 1976; Katz and Miledi 1968). However, a reduction of external Ca\textsuperscript{2+} may bring about opposite effects on Ca\textsuperscript{2+} influx (Piccolino and Pignatelli 1996; Piccolino et al. 1999). On the one hand, a [Ca\textsuperscript{2+}]\textsubscript{o} decrease tends to reduce Ca\textsuperscript{2+} influx because of the reduced driving force and to maintain the viability of the tissue. In that respect the in vitro retina stands in contrast to dissociated cell preparations where the surrounding liquid equilibrates rapidly with the superfusion medium. Retina may also differ from more conventional slice preparations where the accessibility of the extracellular space can be favored by limited tissue thickness and by the possible alterations of the extracellular space resulting from experimental procedures.

There is abundant experimental evidence indicating that a slow and incomplete equilibration occurs between the extracellular space and the superfusing medium in an eyecup preparation. For instance although glutamate is effective at micromolar concentrations for stimulating dissociated horizontal cells of the turtle retina, its concentration must be raised to tens of millimolar to affect the same cells in the eyecup (Ariel et al. 1984; Cervetto and MacNichol 1972; Golard et al. 1992; Ishida and N eyton 1985). Intraretinal diffusion barriers and powerful uptake mechanisms may prevent the penetration of glutamate down to the more deep (scleral) layers of retinal tissue (Miyachi 1988; Normann et al. 1986). Diffusion problems and uptake mechanisms also may limit the study in the intact retina of neurotransmitters, as for instance GABA (see Yazulla 1991).

Difficulty in diffusion must be taken particularly into account when attempting to change the ionic composition of the medium. This is especially the case for divalent cations because of the existence on the external surfaces of neuronal membranes of an excess of negative charges that tends to sequester the ions and limit their mobility in the extracellular compartment (Harsanyi and Mangel 1993; McLaughlin 1989; Morris and Krnjevic 1981; Piccolino and Pignatelli 1996; Piccolino et al. 1999). There are several indications that divalent cation diffusion in both retina and in more conventional slice preparations may be slow and equilibrium may not be achieved in the conditions of the experiment (Hegstad et al. 1989; Morris and Krnjevic 1981; Piccolino et al. 1996).

Diffusion problems acquire a particular relevance for the interpretation of experimental results in situations in which the extracellular Ca\textsuperscript{2+} level is reduced to reduce Ca\textsuperscript{2+} influx through the voltage-dependent Ca\textsuperscript{2+} channels of the plasma membrane. This is commonly done, for instance, to block chemical synaptic transmission (Cervetto and Piccolino 1974; Kaneko and Shimazaki 1976; Katz and Miledi 1968). However, a reduction of external Ca\textsuperscript{2+} may bring about opposite effects on Ca\textsuperscript{2+} influx (Piccolino and Pignatelli 1996; Piccolino et al. 1999). On the one hand, a [Ca\textsuperscript{2+}]\textsubscript{o} decrease tends to reduce Ca\textsuperscript{2+} influx because of the reduced driving force and

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availability of Ca\(^{2+}\) ions at the channel mouth. On the other hand, because of the reduced screening of the negative charges on the external surface of plasma membrane, \([\text{Ca}^{2+}]_o\) decrease may result in an increased opening of Ca\(^{2+}\) channels and thus an increased Ca\(^{2+}\) influx (Baldridge et al. 1998; Piccolino et al. 1998). Whether Ca\(^{2+}\) influx will ultimately decrease or increase depends on the prevalence of the one or the other of these two contrasting effects.

In recent experiments on the eyecup preparation of the turtle retina, it was found that decreased \([\text{Ca}^{2+}]_o\) could result in an increase of transmitter release from photoreceptors via a surface-charge-mediated augmentation of Ca\(^{2+}\) influx in low-Ca\(^{2+}\) media (Piccolino et al. 1996; see also Baldridge et al. 1998). On the basis of this, we suggested that the persistence, or enhancement, of synaptic transmission during the application of low-Ca\(^{2+}\) media, as observed in previous studies (Hanksins et al. 1985; Harsanyi and Mangel 1993; Piccolino et al. 1996; Rowe 1987; Schwartz 1986; Umino and Watanabe 1987), does not necessarily imply that transmitter release from photoreceptor terminals is Ca\(^{2+}\) independent.

There was, however, a difficulty with our interpretation. An increase of Ca\(^{2+}\) current and of intracellular Ca\(^{2+}\) level in isolated photoreceptors was observed only with relatively modest reductions of extracellular Ca\(^{2+}\) (0.3–1.0 mM), whereas synaptic transmission enhancement in the superfused eyecup was seen even when Ca\(^{2+}\) was omitted from the perfusing medium (resulting in Ca\(^{2+}\) concentrations in the micromolar range) or was reduced to extremely low levels (in the nanomolar range) by using Ca\(^{2+}\) buffers. To account for this apparent inconsistency, we assumed that in our preparation \([\text{Ca}^{2+}]_o\) would remain at levels capable of supporting classical synaptic transmission (hundreds of micromolar), even after prolonged application of low-Ca\(^{2+}\) media, due to incomplete equilibration of divalent cation concentrations between perfusing medium and extracellular compartment near the photoreceptor synapse.

The aim of the present work is to provide an experimental test of our hypothesis. We have measured Ca\(^{2+}\) concentrations in the extracellular space of the retina by using ion-sensitive electrodes in conditions similar to those used in the study of the effects of low-Ca\(^{2+}\) media on synaptic transmission in the retina. Our results show that, in an eyecup, extracellular Ca\(^{2+}\) does not faithfully follow the changes in the superfusing medium but remains at elevated levels even after prolonged perfusion with low-Ca\(^{2+}\) media. The hypothesis that low-Ca\(^{2+}\) media can enhance synaptic transmission from photoreceptor to horizontal cells by promoting Ca\(^{2+}\) entry into photoreceptor synaptic terminals therefore is supported.

**METHODS**

**Preparation**

Eyecups were prepared from the retina of turtles, *Pseudemys scripta elegans*, obtained from William A. Lemberger (Oshkosh, WI) and kept in outdoor ponds. The animals were killed by decapitation and pithed after being anesthetized with ketamine (200 mg/kg). Details of the experimental methods used to remove the eye and preparing the eyecup are given elsewhere (Piccolino et al. 1984). The preparation was superfused with a saline of the following composition (in mM/l): 110 NaCl, 2.6 KCl, 22 NaHCO\(_3\), 2 MgCl\(_2\), 2 CaCl\(_2\), and 10 D-glucose. This solution was bubbled continuously with a mixture of 95% O\(_2\)-5% CO\(_2\), to a final pH of 7.4. In some experiments, to assess the viability of the preparation, we recorded intracellularly the light responses of retinal neurons (usually horizontal cells) by using fine-tip glass micropipettes prepared with a Brown-Flaming puller (Model P-77. Sutter Instrument, Novato, CA) filled with 3 M potassium acetate and 0.2 M KCl (resistance 150–500 MΩ).

**Ca-sensitive microelectrodes**

The extracellular concentration of Ca\(^{2+}\) was measured with double-barrelled ion-sensitive microelectrodes based on the Calcium Ionometer I-Cocktail A by Fluka (Switzerland, No. 21048). The microelectrodes were pulled with the P-77 Brown-Flaming puller using double-barrelled borosilicate glass “theta” capillaries (WPI, Sarasota, FL, No. TST150–6). After pulling, the barrel to be used as the reference electrode was backfilled with distilled water. Then several micropipettes were exposed for 4–16 h at room temperature to an atmosphere of silane obtained by dropping a few milliliters of a solution of dimethyldichlorosilane in carbon tetrachloride (10% in volume, both from Sigma) in a tightly closed jar. After silanization the active barrel was backfilled with 0.5 μL of the ion exchange solution. The tip of the microelectrode then was broken gently on a piece of soft paper, thus allowing the ion exchanger to move spontaneously to the very tip. Finally the distilled water in the reference barrel was replaced, by backfilling, with a solution containing (in mM) 140.0 NaCl, 5.0 KCl, 2.6 CaCl\(_2\), and 5.0 Tris (pH 7.5). The same solution also was used for filling up the ion selective barrel away from the tip. Typically the microelectrodes used in the experiments had tips with diameters of 10–15 μm.

**Solutions and calibration of Ca-selective microelectrodes**

The relation between the potential measured by the ion-sensitive electrodes and the ionic concentration in the medium is described by the Nicolsky equation (Amman 1986; Amman et al. 1983), which takes into account the contribution to the measured potential of the ion investigated (Ca\(^{2+}\) in our case) and the interference by the other ions present in the solution

\[ E = \left( \frac{RT}{F} \right) \ln (a_i + k_{ij} a_j) \]

where \(E\) is the potential, \(R\) is universal gas constant, \(T\) is absolute temperature, \(F\) is Faraday’s electrochemical equivalent, \(a_i\) and \(a_j\) are the ion activities, and \(z_i\) and \(z_j\) are the valence of the ions. In this equation, \(k_{ij}\) represents the interference between the ions, the index \(i\) indicates the ion being sensed, and index \(j\) indicates the interfering ion. Although the notion of ion selectivity implies that a particular ion has the largest influence on the electrode potential, the contribution of other ions cannot be ignored, especially when the concentration of these ions is much higher than the concentration of the ion being investigated. We measured Ca\(^{2+}\) concentrations ranging from 2 × 10\(^{-5}\) M to 10\(^{-9}\) M in the constant presence of >10\(^{-1}\) M Na\(^+\), thus interference from at least one cation (Na\(^{+}\)) began to be significant and responsible for a nonlinear dependence of the potential from log \([\text{Ca}^{2+}]_o\) when \([\text{Ca}^{2+}]_o\) drops to <1 μM. The solutions used for calibration had about the same ionic concentration as the solution employed in experiments. The base calibration solution was close to Ringer solution for turtle and included (in mM) 140 NaCl, 2.6 KCl, and 5 Tris (pH 7.5). Three different concentrations of CaCl\(_2\) were used: 2 mM and 60 and 2 μM. An additional calibration solution, used for experiments in which EGTA was present in the perfusing solution, was made by buffering \([\text{Ca}^{2+}]_o\) to 1 nM with the addition of 5 mM EGTA and appropriate amounts of CaCl\(_2\) and MgCl\(_2\) (calculated by using the computer program “Sol.i.d.” written by Eric Ertel (Hoffman-LaRoche, Basel). Data were discarded if there were inconsistencies between calibration readings obtained before penetrating the tissue and after pulling up the electrode. As generally occurs with
ion-sensitive electrode experiments, penetration of the retina with relatively large-tipped electrodes might have mechanically distorted the tissue and somewhat interfered with ionic measurements. The overall reliability of the procedure, however, was supported by the observation that the same concentrations were measured, during the application of low-Ca²⁺ media, after having pulled up the electrode completely from the tissue and then reinserted it to the same depth, in the same or different location.

RESULTS

Effects of low-Ca²⁺ medium on [Ca²⁺]₀ in the retina

We perfused the retina with a nominally Ca²⁺-free solution and measured the extracellular Ca²⁺ concentration within the retina tissue. Figure 1 illustrates the results. With the electrode in the perfusion fluid, above the vitreal side of the retina, the perfusion solution was changed to a Ca²⁺-free Ringer. The concentration of Ca²⁺ started decreasing after ~1 min after the application of the test solution, and within ~3 min Ca²⁺ reached a level a little higher than 2 μM, which obviously corresponded to that of the test medium (Fig. 1A). On reapplying the normal solution, Ca²⁺ level rapidly reached the control value. Thereafter the electrode was advanced into the retinal tissue to a point 130 μm below the vitreal surface so that its tip resided at the level at which horizontal cells normally are penetrated with intracellular microelectrodes. After the application of test solution (Fig. 1B), Ca²⁺ began to decrease with a slow time course that reached, after 30 min, a value corresponding to 1.4 mM. In nine experiments in which extracellular Ca²⁺ was measured at retinal depths of 100–200 μm, the average level of [Ca²⁺]₀ reached after 30-min application of Ca²⁺-free Ringer was 1.41 mM ± 0.33 (mean ± SD). In some experiments, low-Ca²⁺ application was conducted for longer periods (from 40 to 60 min), and even in these circumstances, [Ca²⁺]₀ never fell <1 mM.

Extracellular [Ca²⁺]₀ as a function of retinal depth

The values of extracellular Ca²⁺ concentration changes induced by the application of low-Ca²⁺ media depended on retinal depth and were substantially lower when the electrode was positioned less deep in the retinal tissue. A typical experiment is illustrated in Fig. 2 where the final level of [Ca²⁺]₀ brought about by the application of low-Ca²⁺ medium ranged from 1.70 at 200 μm depth to 0.23 at 60 μm. This suggested that a substantial Ca²⁺ gradient exists vertically across the retinal tissue in the presence of low-Ca²⁺ media, a view verified by experiments in which electrode position was changed during prolonged application of low-Ca²⁺ media. One of these experiments is illustrated in Fig. 3 where a medium lacking Ca²⁺ was applied for ~30 min while the electrode was deep in the retinal tissue (~100 μm below the vitreal surface) leading to a final concentration of ~1.05 mM at that electrode position. Afterward the electrode tip was raised by moving the microelectrode vertically to different levels in step fashion (the size of each step is indicated by the numbers near the downward pointing arrows). The measured concentration decreased rather abruptly after each movement and became similar to that of the

FIG. 1. Measurement of [Ca²⁺]₀ changes induced by application of a nominally Ca²⁺-free saline in the eyecup preparation of the turtle retina while the tip of the ion-sensitive electrode was in the perfusing solution over the retina (A) and in the retinal tissue at ~130 μm below the vitreal surface (B). In B the minimal level of [Ca²⁺]₀ measured by the electrode inside the retina at the end of 30-min application was estimated to be ~1.4 mM.

FIG. 2. [Ca²⁺]₀ changes induced by 30-min application of a nominally Ca²⁺-free saline at 3 different depths of the retinal tissue (as indicated in μm at left). Final level of [Ca²⁺]₀, attained at the different electrode positions is indicated (below each tracing). †, beginning and end of the application of the test solution.

In the retina

FIG. 3. Changes of Ca²⁺ concentration during superfusion with the Ca²⁺-free saline with the electrode initially positioned at 100 μm depth (relative to the vitreal surface) and afterward withdrawn out of the retina in step fashion. †, beginning and the end of the application of the Ca²⁺-free solution. Approximately 30 min after the beginning of test solution application, the microelectrode was withdrawn in 4 subsequent steps. Each step is marked (↓) with the adjacent numbers indicating the size of the electrode withdrawal movement.
increase of Ca\(^{2+}\) depth. [Ca\(^{2+}\)]\(_o\) in extracellular space was measured after 30 min of superfusion with nominally Ca\(^{2+}\)-free solution together with 0.4 mM Zn\(^{2+}\). The final Ca\(^{2+}\) levels measured in both the perfusing fluid and inside the retina did not significantly differ from those observed in the absence of the exogenous antagonists. This is illustrated in Fig. 4, which compares the levels reached by [Ca\(^{2+}\)]\(_o\) at different retinal depths after 30 min application of a nominally Ca\(^{2+}\)-free medium containing 0.4 mM Zn\(^{2+}\) with the levels reached after application of a Ca\(^{2+}\)-free medium lacking Zn\(^{2+}\). There appears to be no significant difference between the two sets of data. Moreover, in spite of the dispersion of data that blurs the relation between depths and concentration, it seems that the Ca\(^{2+}\) gradient is steeper in the proximal part of the retina.

In the previous work in which a surface-charge-mediated increase of Ca\(^{2+}\) influx was produced by decreasing [Ca\(^{2+}\)]\(_o\) the low-Ca\(^{2+}\) media generally were applied in the presence of divalent cations having a strong influence on surface charges (Baldridge et al. 1998; Piccolino et al. 1996). To simulate such conditions, in some experiments the retina was perfused for 15 min with 0.4 mM Zn\(^{2+}\) (\(n = 6\)) before applying a nominally Ca\(^{2+}\)-free solution together with 0.4 mM Zn\(^{2+}\). The final Ca\(^{2+}\) levels measured in both the perfusing fluid and inside the retina tissue did not significantly differ from those observed in the absence of the exogenous antagonists. This is illustrated in Fig. 5, which compares the levels reached by [Ca\(^{2+}\)]\(_o\) at different retinal depths after 30 min application of a nominally Ca\(^{2+}\)-free medium containing 0.4 mM Zn\(^{2+}\) with the levels reached after application of a Ca\(^{2+}\)-free medium lacking Zn\(^{2+}\). There appears to be no significant difference between the two sets of data. Moreover, in spite of the dispersion of data that blurs the relation between depths and concentration, it seems that the Ca\(^{2+}\) gradient is steeper in the proximal part of the retina.

**Effects of low-Ca\(^{2+}\) medium containing EGTA**

In some experiments, we measured [Ca\(^{2+}\)]\(_o\) at various levels of retina during the application of media in which Ca\(^{2+}\) had been buffered to very low levels (1 nM) by adding 5 mM EGTA and appropriate concentrations of CaCl\(_2\). In the experiment illustrated in Fig. 5, the electrode initially was positioned at a depth of 100 \(\mu\)m with respect to the vitreal surface. After the application of low-Ca\(^{2+}\) media containing EGTA, the level of Ca\(^{2+}\) inside the retina dropped to lower levels than seen in the absence of the buffer. For instance, after the initial 30-min application of the test solution, [Ca\(^{2+}\)]\(_o\) at 100 \(\mu\)m depth dropped to 0.51 mM, a level never reached with low-Ca\(^{2+}\) media lacking EGTA. Nevertheless, Ca\(^{2+}\) still remained much higher than in the superfusion medium and well above the level at which Ca\(^{2+}\) activates transmitter release inside synaptic terminals (1–100 nM) (see Adler et al. 1991; Heidelberger et al. 1994; Matthews 1996; Rieke and Schwartz 1996). In similar experiments in which extracellular Ca\(^{2+}\) was measured at retinal depths of 110–200 \(\mu\)m, during the application of low-Ca\(^{2+}\) solutions containing 5 mM EGTA, the level of [Ca\(^{2+}\)]\(_o\) reached after 30 min never fell <0.30 mM (0.48 ± 0.18; mean ± SD, \(n = 5\)). Moreover as in the case of perfusion with nominally Ca\(^{2+}\)-free media lacking EGTA, large vertical gradients of Ca\(^{2+}\) existed in the retina during the application of media containing Ca\(^{2+}\) buffers. This appeared clearly when the electrode was pulled up in step fashion out of the tissue (the size of the steps is indicated in Fig. 5 near the down arrow).

**DISCUSSION**

The results of the present experiments can be summarized by saying that the Ca\(^{2+}\) concentration in the more scleral layers of the retina eyecup preparation remains substantially elevated after prolonged superfusion of the vitreal surface with low-Ca\(^{2+}\) media. In particular at the depths where horizontal cells are commonly encountered with intracellular electrodes, [Ca\(^{2+}\)]\(_o\) never decreased to <1 mM after 30- to 60-min application of nominally free Ca\(^{2+}\) media, and it remained at values >0.3 mM even after applications of media containing EGTA that buffered Ca\(^{2+}\) concentration in the nanomolar range.

**FIG. 5. Changes of Ca\(^{2+}\) concentration during superfusion with EGTA-buffered solution ([Ca\(^{2+}\)] = 1 nM) and after the subsequent withdrawing of microelectrode out of retina in step fashion. Microelectrode initially was positioned in retina at the depth of 100 \(\mu\)M relative to the vitreal surface. ↑ moment of the application of EGTA-solution. Approximately 30 min after the beginning of test, solution application the microelectrode was withdrawn in 5 steps. Each step is marked (↓) with the adjacent numbers indicating the size of displacements relative to the initial electrode position.**
In general a large concentration difference in the retina preparation between superfusing medium and extracellular space may depend on the integrity of the thin and tortuous extracellular space of the retina, with intimate contact between neurons and glial cells, on the existence of diffusion barriers from both the vitreal and scleral side and on the presence of powerful mechanisms of transport on the cell membrane of both neurons and glia. These elements combine to maintain the constancy of the extracellular microenvironment and to reduce the possible influences of changes in the composition of the superfusing medium (Dreher et al. 1988; Karwoski et al. 1989; Linsenmeier and Steinberg 1983; Stone et al. 1995; Tout et al. 1993). It is indeed well known that retina is able to maintain extracellular ion concentrations at levels that differ from those of the superfusion solutions. For instance, it was shown in various in vitro preparations from different animals that the extracellular concentration of K\(^+\) in subretinal space (i.e., between photoreceptors and retinal pigment epithelium) is about two times higher than in the superfusion medium (Bykov et al. 1984; Dick and Miller 1985; Dick et al. 1985; Dmitriev and Bykov 1990; Miller and Steinberg 1982; Oakley and Green 1976). Moreover, measurements in intact cat demonstrated that there are concentration gradients in vitreous and across the retina for K\(^+\) (Steinberg et al. 1980) and for Ca\(^{2+}\) (Gallermore et al. 1994). Recently it was found that retinal extracellular pH was substantially different from the pH of the superfusion solution in isolated fish retina (by 0.23 pH units) (see Dmitriev and Mangel 1997) and rabbit eyecup (by 0.62 pH units) (see Dmitriev and Mangel 1998).

As already mentioned, a limited diffusion of Ca\(^{2+}\) may depend also on the presence of an excess of Ca\(^{2+}\) on the external surface of neuronal membrane due to electrostatic interactions with the negative surface charges (McLaughlin 1989; Morris and Krnjevic 1981; Piccolino et al. 1999). This excess of Ca\(^{2+}\) may act as a functional reserve capable of effectively buffering the concentration of the ions in the bulk extracellular medium and thus seriously limiting any attempt to change it via manipulation of the perfusing medium (Piccolino and Pignatelli 1996; Piccolino et al. 1999). Moreover, the difficulty in modifying [Ca\(^{2+}\)]\(_o\) in the deep retinal layers may depend on the characteristics of Ca\(^{2+}\) metabolism in the photoreceptor layer and, in particular, on the intense exchange of Ca\(^{2+}\) between extracellular and intracellular compartment because ~15% of dark current is carried by Ca\(^{2+}\) (McNaughton 1995; Yau and Bykov 1989). This roughly equals 10\(^7\) Ca\(^{2+}\) ions per second, a rate such that all extracellular Ca\(^{2+}\) would be exchanged in ~1 min. Because the total intracellular Ca\(^{2+}\) in photoreceptors is >10 times higher than the content of extracellular space (taking into account the volume occupied by cells), it is easy to envision why Ca\(^{2+}\) losses from the extracellular space could be compensated for easily in the distal retina.

Even after prolonged application of low-Ca\(^{2+}\) media in the retina eyecup preparation, the deep retinal layers still retain a sufficient [Ca\(^{2+}\)]\(_o\) to provide a large driving force for Ca\(^{2+}\) entry in nerve cells via the Ca\(^{2+}\) channels of the plasma membrane. This is still the case for EGTA-containing media because [Ca\(^{2+}\)]\(_o\) did not fall <0.30 mM in the distal retina. In general Ca\(^{2+}\) concentration in the presynaptic terminals of chemical synapses must rise at concentrations of ~20–100 \(\mu\)M to activate transmitter release (Adler et al. 1991; Heidberger et al. 1994; Matthews 1996), and in photoreceptor synapses the release is likely to occur even at concentrations of <1 \(\mu\)M (Krizaj and Copenhagen 1998; Rieke and Schwartz 1996). As a matter of fact, experiments carried out on isolated photoreceptors of the salamander retina show that reducing [Ca\(^{2+}\)]\(_o\) to values in the range of 0.3–1.0 mM actually may result in an increase of Ca\(^{2+}\) current at the physiological membrane potential of photoreceptors in darkness (~35, ~30 mV). This effect is due to a shift to the left (i.e., toward less depolarized potentials) of the Ca\(^{2+}\) current activation curve (Baldridge et al. 1998; Piccolino et al. 1996), which in turn results from a decrease of the neutralizing action of Ca\(^{2+}\) ions on the negative charge at the external surface of the plasma membrane. The shift more than compensates for the reduced driving force due to decreased [Ca\(^{2+}\)]\(_o\), and thus may lead to an increase of [Ca\(^{2+}\)]\(_i\) in photoreceptor terminals compared with the control conditions.

On the basis of our results it is therefore easy to understand why the application of low-Ca\(^{2+}\) media to the retina may lead to an enhancement, rather than to a depression, of synaptic transmission from photoreceptor endings (Harsanyi and Mangel 1993; Rowe 1987; Piccolino et al. 1996). In particular, it can be understood why this applies also to experiments involving the application of superfusing media with Ca\(^{2+}\) concentrations so low that they would not support any transmitter release when applied to isolated cell preparations.

Combined with the results of other studies of ionic manipulation effects on synaptic transmission from photoreceptors to horizontal cells, the present study appears to invalidate the hypothesis that synaptic transmission at this level is Ca\(^{2+}\) independent (Schwartz 1986). This hypothesis was based on the observation that synaptic transmission from photoreceptors to second-order neurons in the presence of a superfusing medium only lacking Ca\(^{2+}\) and containing Co\(^{2+}\) ions. Interpreting this observation, it was assumed that no significant Ca\(^{2+}\) influx could occur into presynaptic terminals in a situation in which Ca\(^{2+}\) channels probably were blocked due to the presence of Co\(^{2+}\). And, moreover, the Ca\(^{2+}\) gradient was minimized as a consequence of the reduced concentration of external Ca\(^{2+}\). This interpretation largely depended on the assumption that the concentration of divergent cations in the extracellular space of the retina followed closely that of the perfusing medium. Later experiments indicated that this was likely not the case (Piccolino et al. 1996). First it appeared that in the eyecup preparation experiments the synaptic blocking effect of Co\(^{2+}\) and of other antagonist divergent cations such as Zn\(^{2+}\) or Ni\(^{2+}\), could be accounted for largely by a shift to the right of the channel activation curve brought about by concentrations of the divergent cations in the extracellular space of the retina that are much lower than those in the bathing medium. Because of this shift (which in turn resulted from an increased screening of the negative surface charges on the plasma membrane), there was a drastic decrease of the Ca\(^{2+}\) current at the physiological membrane potential of photoreceptors, i.e., at the dark potential. In these conditions, lowering [Ca\(^{2+}\)]\(_o\) would bring about an increase of Ca\(^{2+}\) current via a shift to the left of the activation curve due to the reduction of surface charge screening. On these grounds, the relative synaptic blocking inefficacy of low-Ca\(^{2+}\) media containing Co\(^{2+}\) (and other divergent antagonists) could be accounted for easily. Moreover, it was also
possible to explain why the synaptic blocking efficacy of Co\(^{2+}\), Zn\(^{2+}\), and Ni\(^{2+}\) was stronger when a normal Ca\(^{2+}\) concentration was present in the perfusing medium than when Ca\(^{2+}\) was omitted from the bathing solution (Cervetto and Piccolino 1974; Piccolino et al. 1996; Schwartz 1986).

In the context of the interpretation of the effects of low-Ca\(^{2+}\) media on synaptic transmission in the retina, the main new information provided by the present results is that perfusing the retina eyecup preparation with nominally zero Ca\(^{2+}\) solutions, or with an EGTA-Ca\(^{2+}\) Ringer in which [Ca\(^{2+}\)]\(_{in}\) is estimated to be \(\sim 1\) nM, causes a relatively small decrease of [Ca\(^{2+}\)]\(_{in}\) in the deep layers of the retina. That lowered level of [Ca\(^{2+}\)]\(_{in}\) is well within the range of the [Ca\(^{2+}\)]\(_{in}\) values capable of promoting Ca\(^{2+}\) influx into photoreceptors at their physiologically relevant potential (Krizaj and Copenhagen 1998; Rieke and Schwartz 1996). A strong support to the notion that synaptic transmission from photoreceptors to horizontal cells is indeed Ca\(^{2+}\)-dependent comes from a recent work carried out in the Xenopus retina showing the existence of a fine tuning among the physiologically relevant presynaptic potential, Ca\(^{2+}\)-cur- tain activation curve, and glutamate release (Witkovsky et al. 1997).

It is worth mentioning here that, as in other regions of the nervous system, the concentration of Ca\(^{2+}\) in the deep layers of the retina may vary in physiological conditions, such as during light-dark adaptation (Bykov et al. 1985; Gallemore et al. 1994; Gold and Korenblat 1980; Livsey et al. 1990; Yoshikami et al. 1980). It is thus possible that changes of [Ca\(^{2+}\)]\(_{in}\) may serve to modulate the transmission process at the photoreceptor synapse in physiological conditions.

In a more general context, the presence of a significant difference between the levels of Ca\(^{2+}\) in the deep extracellular layers of an in vitro nervous preparation and the perfusing medium, even after a prolonged application of low-Ca\(^{2+}\) media, invites caution in interpreting as Ca\(^{2+}\)-independent any process that persists after prolonged application of low-Ca\(^{2+}\) solution. This is particularly important for thick tissue preparations (wedges or thick slices), especially when the morphological and functional integrity of extracellular space is preserved, and when diffusion barriers are likely to exist between the perfused surface and the layer of nervous tissue under study (Nicholson 1980; Nicholson and Hounsagrd 1983; Toot et al. 1993). Of course, the interpretation of the results of low-Ca\(^{2+}\) experiments is more straightforward in the case of preparations in which diffusion is less restricted by morphological barriers (e.g., the isolated retina where free diffusion occurs from the photoreceptor side) and in particular for isolated cell preparations where the membrane surface is readily accessible to the perfusing medium. On these grounds, it is likely that a true Ca\(^{2+}\)-independent process operates in the release of synaptic transmitter from retinal horizontal cells (Schwartz 1987).

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