Mechanism of Generation of Spontaneous Miniature Outward Currents (SMOCs) in Retinal Amacrine Cells

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Abstract: A subtype of retinal amacrine cells displayed a distinctive array of K⁺ currents. Spontaneous miniature outward currents (SMOCs) were observed in the narrow voltage range of −60 to −40 mV. Depolarizations above approximately −40 mV were associated with the disappearance of SMOCs and the appearance of transient (Iᵢₒ) and sustained (Iᵢₑ) outward K⁺ currents. Iᵢₒ appeared at about −40 mV and its apparent magnitude was biphasic with voltage, whereas Iᵢₑ appeared near −30 mV and increased linearly. SMOCs, Iᵢₑ, and a component of Iᵢₒ were Ca²⁺ dependent. SMOCs were spike shaped, occurred randomly, and had decay times appreciably longer than the time to peak. In the presence of cadmium or cobalt, SMOCs with pharmacologic properties identical to those seen in normal Ringer’s could be generated at voltages of −20 mV and above. Their mean amplitude was Nernstian with respect to [K⁺]ₑ, and they were blocked by tetraethylammonium. SMOCs were inhibited by iberiotoxin, were insensitive to apamin, and eliminated by nominally Ca²⁺-free solutions, indicative of BK-type Ca²⁺-activated K⁺ currents. Dihydropyridine Ca²⁺ channel antagonists and agonists decreased and increased SMOC frequencies, respectively. Ca²⁺ permeation through the kainic acid receptor had no effect. Blockade of organelle Ca²⁺ channels by ryanodine, or intracellular Ca²⁺ store depletion with caffeine, eradicated SMOCs. Internal Ca²⁺ chelation with 10 mM BAPTA eliminated SMOCs, whereas 10 mM EGTA had no effect. These results suggest a mechanism whereby Ca²⁺ influx through L-type Ca²⁺ channels and its subsequent amplification by Ca²⁺-induced Ca²⁺ release via the ryanodine receptor leads to a localized elevation of internal Ca²⁺. This amplified Ca²⁺ signal in turn activates BK channels in a discontinuous fashion, resulting in randomly occurring SMOCs.

Key words: potassium channels • calcium-induced calcium release • ryanodine receptors • L-type calcium channels • ganglion cells

Introduction

In the vertebrate retina, amacrine and ganglion cells reside two synapses away from the photoreceptors. These third order neurons are driven by glutamatergic inputs from bipolar cells, and are the first neurons in the visual pathway that generate action potentials (Dowling and Werblin, 1969; Werblin and Dowling, 1969; Kaneko, 1970). Perhaps, because of their distinctive nonlinear membrane properties, these neurons have a large assortment of K⁺ channels. We explored the properties and relationships between several K⁺ channels found in one subtype of third order neuron: an amacrine cell subtype.

K⁺ channels can be broadly categorized based on gating into voltage- and ligand-activated subtypes (Hille, 1992). A notable group amongst the ligand-activated are those gated by intracellular Ca²⁺, the Ca²⁺-activated K⁺ channels (I}$/H11001$, and their presence in retinal third order neurons has been documented (Lipton and Tauck, 1987; Latorre et al., 1989; Vergara et al., 1998; Wang et al., 1998). Cytoplasmic calcium ([Ca²⁺]i) responsible for the gating can arise either via influx through plasmalemmal channels or efflux from the ER (Zorumski et al., 1989; Kostyuk and Verkhratsky, 1994; Wigirda and Dryer, 1994; Davies et al., 1996; Berridge, 1998; Marrion and Tavalin, 1998; Hurley et al., 1999; Imaizumi et al., 1999). The release of organelle Ca²⁺ is mediated primarily via two subtypes of intracellular Ca²⁺ channels. While both these channels are calcium sensitive, one subtype releases calcium in response to elevations in the levels of inositol 1,4,5-trisphosphate (IP₃) and the other responds primarily to elevations of cytoplasmic Ca²⁺ by Ca²⁺-induced Ca²⁺ release (CICR)* (Fabiato, 1983; Berridge, 1993, 1998). The latter is sensitive to the plant alkaloid ryanodine, and is thus called the ryanodine receptor (RyR; Pozzan et al., 1994; Sutko and Airey, 1996; Zucchi and Ronca-Testoni, 1997). CICR is extremely well documented in cardiac muscle and also found in neurons (Kuba, 1994; Cheng

*Abbreviations used in this paper: CICR, Ca²⁺-induced Ca²⁺ release; DHP, dihydropyridine; KA, kainic acid; RyR, ryanodine receptor; SMOC, spontaneous miniature outward current; STOC, spontaneous transient outward current; TTX, tetrodotoxin; VGCC, voltage-gated calcium channel.

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et al., 1996; Verkhratsky and Shmigol, 1996; Cohen et al., 1997; Jacobs and Meyer, 1997; Usachev and Thayer, 1997). It is thought to amplify calcium signals, such as those originating from influx via voltage-gated calcium channels (VGCCs) (Friel and Tsien, 1992). This amplified [Ca^{2+}] signal may rise to magnitudes of several μM in local regions, activating membrane proteins such as Ca^{2+}-activated K⁺ or Cl⁻ channels (Imaizumi et al., 1998; Gordienko et al., 1999). These CICR-generated localized elevations of [Ca^{2+}], called “Ca^{2+} sparks,” have been detected in smooth as well as cardiac muscle and there is evidence that sparks can activate clusters of large conductance K_{Ca} channels (BK subtype) (Nelson et al., 1995; Imaizumi et al., 1998; ZhuGe et al., 1999). In muscle cells under voltage clamp, K⁺ fluxes through BK channels generated in this manner appear as random outward currents termed spontaneous transient outward currents (STOCs) (Benham and Bolton, 1986; Bolton and Imaizumi, 1996). STOCs are thought to govern the resting membrane potential in smooth muscle (Nelson et al., 1995; Jaggar et al., 1998). A similar mechanism, if existent in excitable neurons such as retinal amacrine cells, can potentially modulate neuronal excitability.

This study characterizes a subtype of retinal amacrine cell with respect to its K⁺ current signature. In addition to voltage-activated K⁺ currents, these neurons display a set of K_{Ca} currents with different temporal properties. These currents activate sequentially as the membrane potential of the neuron depolarizes. The K_{Ca} currents consist of a randomly occurring transient outward current, an early onset transient outward current, and a sustained outward current. The random outward current has properties similar to the STOCs seen in muscle cells, and in neurons are called spontaneous miniature outward currents (SMOCs) (Hartzell et al., 1977; Mathers and Barker, 1981, 1984; Satin and Adams, 1987; Fletcher and Chiappinelli, 1992; Merriam et al., 1999; Arima et al., 2001). This study reports the presence of SMOCs in retinal neurons and elucidates their mechanism of generation. A preliminary description of this work was presented by Mitra and Slaughter (2000).

MATERIALS AND METHODS

Retinal Cell Preparation

Aquatic tiger salamanders (Ambystoma tigrinum) obtained from Kons Scientific and Charles Sullivan were kept in tanks maintained at 4°C on a 12 h light/dark cycle. Experiments were performed on acutely isolated neurons by enzymatic dissociation of the retina (Lam, 1972; Bader et al., 1979; Pan and Slaughter, 1995). All procedures were performed in accordance with the United States Animal Welfare Act and the National Institutes of Health Guide for the Care and Use of Laboratory Animals (publication 85-23), and were approved by the University Animal Care Committee. Briefly, the animals were decapitated, pithed, and the eyes were enucleated. The cornea, lens, and vitreous were removed and the retina was isolated by separating it from the pigment epithelium. During this process the entire tissue was continuously immersed in normal amphibian Ringer’s solution. The isolated retina was transferred to 350 μl of enzyme solution containing papain (12 U/ml; Worthington Biochemicals), deoxyribonuclease (0.28 mg/ml; Worthington Biochemicals), 5 mM L-cysteine, 1 mM EDTA dissolved in normal amphibian Ringer’s, and adjusted to pH 7.4. The tissue was digested in enzyme solution for 40 min at room temperature and continuously oxygenated. The retina was rinsed five times with normal amphibian Ringer’s solution, then gently shaken until the entire tissue dissociated. The cells were then plated onto lectin (0.4 mg/ml)-coated coverslips, allowed to settle for ~2 min, and then overlaid with normal amphibian Ringer and stored in a 13°C incubator. Experiments were performed within 4 h of dissociation. The cell type used for this study did not show any distinct morphological characteristics. However, they were identified by a distinct current signature exhibited when subjected to voltage clamp step depolarizations in normal Ringer (see Results).

Experimental Setup and Data Acquisition

The plated coverslips were placed in a plexiglass recording chamber having a volume of 350 μl. One end of the chamber housed a suction tube connected to a peristaltic pump. The cells were viewed with an inverted microscope equipped with a 40× lens. Retinal cells were constantly superfused at room temperature with normal amphibian Ringer’s solution bubbled with oxygen using a gravity fed perfusion system. The tubes of the gravity-fed perfusion system were connected to a common manifold, which held a wide bore patch pipette. This patch pipette was placed close to the cell during recordings, and solution exchanges could be achieved within 2–3 s. Control and test solutions were applied using this system. The drug effects described in this paper refer to steady-state effects usually obtained after 10–30 s of drug application. Data were acquired with an Axopatch-1C patch clamp amplifier (Axon Instruments, Inc.). analogue signals were filtered at 5 KHz and sampled at 20 KHz with the DigiData 1200 analogue-to-digital board (Axon Instruments, Inc.) using Clampex 8 software. Data were acquired using the whole cell variant of the patch clamp technique, in both the continuous voltage and current clamp modes (Hamill et al., 1981). Patch pipettes were fashioned from borosilicate glass and had resistances of ~5 MΩ when measured in bath solution. Data shown in this paper were corrected for pipette junction potential (~10 mV). Except for the voltage values given in Fig. 2, C and D, and associated text, where the voltage error due to series resistance (~10 MΩ) could have an impact on the interpretation, the voltages are uncorrected for series resistance. Since these neurons had high input resistances, leak subtraction was not necessary. This, along with the observation that at relatively negative voltages (~90 to ~30 mV) the clamp currents were very small, suggested that the voltage error due to uncompensated series resistance was insignificant within this range. At more depolarized voltages the error can be significant. However, most of the arguments presented in this and the following paper do not rely on the absolute value of the voltage at these potentials. Cells were held at ~80 mV except when specifically mentioned in the text.

SMOC Analysis

Repeated voltage steps of up to 500 ms duration elicited SMOCs, which were analyzed using Mini Analysis program (version 4.1.1; Synaptosoft, Inc.) and Origin (version 6.0; Microcal Software, Inc.). SMOCs were detected using either the automatic detection mode in the Mini Analysis software or manually by eye. SMOCs
seen in normal Ringer’s appeared on a zero current baseline, whereas those seen at more depolarized levels with cobalt were superimposed on the voltage-activated current. Prior to SMOC detection, the value of the peak-positive deflection of the baseline current noise in the data group was set as a threshold to eliminate SMOCs that are indistinguishable from the baseline noise. Invariably 150–500 SMOCs were analyzed for each parameter. A minimum of 10 s of data from each cell displaying SMOCs were used for analysis of frequencies. Under conditions in which SMOC frequency was greatly reduced, the time was considerably longer. Invariably, each pharmacological manipulation was performed at different test voltages. Irrespective of the test voltage used, results were found to be qualitatively consistent in all cells tested. Average values given are from a subset of this entire dataset, comprising the cells that were examined at the reported voltage. Data are expressed as mean ± SEM. Statistical differences were ascertained by Students t test, where P < 0.05 was deemed significant.

Solutions

Normal amphibian Ringer’s contained (in mM): 111 NaCl, 2.5 KCl, 1.8 CaCl₂, 1 MgCl₂, 10 dextrose, and 5 HEPES, buffered to pH 7.8 with NaOH and oxygenated. Co²⁺ Ringer was similar to normal Ringer’s except that it contained 4–20 mM CoCl₂ and variable amounts of CaCl₂ (0–4.5 mM). Addition or removal of CoCl₂ or CaCl₂ to normal Ringer was accompanied by adjustment of the NaCl concentration to maintain osmolarity. Solutions mentioned as containing “0 mM Ca” contained nominal unbuffered levels of calcium. Since only the bath calcium concentration was manipulated in this study, the notation “Ca²⁺-free” used throughout the text, unless indicated otherwise, refers to external calcium. High K⁺ Ringer’s with or without the addition of Co²⁺ was prepared by equimolar substitution of NaCl with KCl. Recording pipettes contained (in mM): 110 K-gluconate, 5 NaCl, 1 MgCl₂, 5 EGTA, 5 HEPES adjusted to pH 7.4 with KOH. The pipette solution also contained an “ATP-regenerating cocktail” consisting of 4 mM ATP, 20 mM phosphocreatine, and 50 U/ml creatine phosphokinase. As noted in the results, bis (o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA, 10 mM) sometimes replaced EGTA in the internal solution. Ryanodine was diazylated into the neurons by including it in the internal solution. Iberiotoxin, apamin, nifedipine, and S-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA, 10 mM) BayK 8644 were obtained from Research Biochemicals, Inc. All other chemicals used in this study were obtained from Sigma-Aldrich. Dihydropyridines (DHPs) were prepared as 10 mM stock in ethanol and stored at −20°C. When using DHPs, care was taken to prevent exposure to light. Appropriate controls indicated that the final concentrations of ethanol used in this study did not have measurable effects. When using peptide toxins (iberiotoxin or apamin), BSA (0.1% wt/vol) was added to both control and toxin-containing solutions.

R E S U L T S

Electrophysiological Characterization of Cell Type

The neurons used for this study were classified as amacrine cells. They generated tetrodotoxin (TTX)-sensitive Na⁺ action potentials, characteristic of both amacrine and ganglion cells (Werblin and Dowling, 1969). They did not fire spikes in a sustained manner in response to constant current injections, rather the spiking invariably accommodated after the first two or three action potentials, similar to that reported in earlier studies of amacrine cells (Barnes and Werblin, 1986; Eliasof et al., 1987). Moreover, cells in salamander retinal slice that show a similar current signature (see below) lie within the amacrine cell layer (unpublished data). On this basis, the neurons used in this study are tentatively identified as amacrine cells.

Fig. 1 A shows current recordings obtained from such a cell in normal Ringer’s, under the whole cell voltage clamp. The cell was held at −80 mV and depolarized from −90 to 70 mV in 10 mV increments, in pulses of 500 ms. The family of traces obtained illustrates the current signature for this cell type. An inward TTX-sensitive (500 nM) Na⁺ current (marked as I₅ₒ in Fig. 1 A) activates at −30 mV. Large outward currents are activated in response to depolarization. These outward currents are primarily K⁺ fluxes, as they reverse near 0 mV when bath Ringer’s contained high K⁺ equivalent to that in the recording pipette (n = 11; unpublished data). Since these cells have high input resistances, very small currents are seen in the voltage range between −90 and −70 mV. The first induced outward current appears at −60 mV. These currents, termed SMOCs, are spike-shaped and TTX-insensitive and occur within the narrow voltage range of −60 to −40 mV (n = 12). Fig. 1 B shows SMOCs elicited in normal Ringer’s at −50 mV. SMOCs at −50 mV have a mean amplitude of 33 ± 0.5 pA, mean time to peak of 2.8 ± 0.1 ms, and a mean 95% decay time of 5.5 ± 0.1 ms (average of four cells, ~1,000 events). In this set of 1,000 analyzed events, SMOCs appeared at a frequency of 9.8 ± 0.5 Hz and the highest SMOC amplitude was 92 pA.

At −40 mV a transient outward current (Iₒ) appeared along with SMOCs (arrow at −40 mV in Fig. 1 A). Depolarization beyond −40 mV leads to the disappearance of SMOCs. Instead, the outward current trace shows a transient Iₒ along with a sustained outward current (Iₒ). The Iₒ starts to activate at −30 mV. Subsequent depolarizations indicate that the apparent proportion of Iₒ and Iₒ changes. This is shown in Fig. 1 C, wherein outward currents elicited at −20 and 40 mV are shown. The apparent magnitude of the transient Iₒ declines in amplitude (measured as the difference between the peak of Iₒ and the point at which it meets the Iₒ), whereas the sustained component (Iₒ) increases in amplitude as the cell is depolarized. Thus, there is a clear gradation in the appearance of outward current types with voltage. This is shown as a normalized current vs. voltage (I-V) plot in Fig. 1 D (averaged data from eight cells). Depolarization elicits SMOCs within the narrow voltage range of −60 to −40 mV, the Iₒ above −40 mV and the Iₒ above −30 mV, successively. The apparent magnitude of Iₒ initially increases, peaks at −20 mV, and then declines with further depolarization, whereas the Iₒ increases linearly with depo-
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Calcium-dependent Outward Currents

The voltage-induced outward currents have an extracellular Ca²⁺-dependent component. Fig. 2 A shows representative current recordings at 0 mV in normal Ringer’s and in Ringer’s containing 6 mM Co²⁺/0 mM Ca²⁺. Elimination of Ca²⁺ influx abolished Iₙo and reduced Iₗo at all voltages capable of eliciting these currents. The effects were fully reversible on switching back to control Ringer’s. The subtracted trace at 0 mV is shown in Fig. 2 B, which is the Ca²⁺ influx-sensitive component, and is presumably mediated via Kᵥ channels. It includes the entire Iₙo and a component of Iₗo referred to as Iₗo-Ca (n = 8). The residual current in 6 mM Co²⁺/0 mM Ca²⁺ is defined as the Ca²⁺-insensitive, voltage-dependent outward K⁺ current, Iₗo-Ca (Fig. 2 A, lower trace). Fig. 2 C shows a normalized I-V plot for the Iₙo (open circles) and its components, viz: Iₗo (closed diamonds) and Iₗo-Ca (open triangles) (average data from eight cells). As shown in Fig. 2 B, the Iₗo-Ca is obtained by subtraction from the mean values of the Iₙo and Iₗo. The voltage values in Fig. 2, C and D, have been corrected for the drop across the series resistance (≈10 MΩ). The figure shows that beyond 0 mV, both Iₙo and Iₗo increase in a relatively linear fashion, whereas Iₗo-Ca shows a reduction in its slope. Fig. 2 D is a plot of the constituent Iₗo-Ca (open triangles) and Iₗo (closed diamonds) at each test voltage. Both components are expressed as a percentage of the total Iₙo at that particular voltage. Note the decline in the percentage of the Iₗo-Ca component with increasing depolarization. The initial decline is due to the appearance of the Iₗo. However, its percentage continues to decrease at voltages beyond 0 mV; a range in which the Iₗo-Ca I-V plot is relatively linear (see Fig. 2 C), suggesting that the channels comprising the Iₗo-Ca are near maximal activation. This decline of the Iₗo-Ca fraction would be expected if it was dependent on Ca²⁺ influx for its activation, since depolarization would tend to reduce the driving force for Ca²⁺ influx into the cell. At 0 mV, Iₗo-Ca makes up 76.8 ± 2.9% of Iₙo. However, at 30 mV the Iₗo-Ca

**Figure 1.** A subtype of retinal third order neurons shows a voltage-graded spectrum of outward currents. (A) Cell was held at −80 mV and currents were elicited in normal Ringer’s by 500 ms depolarizing voltage steps from −90 to 70 mV in 10 mV increments. The sodium current is shown by arrow marked as Iₙ. Note the appearance of the Iₙo at −40 mV (arrow). The magnification of the figure does not allow outward currents to be discerned between −90 to −50 mV (overlapping traces at these voltages shown by curved arrow). (B) At higher gain, SMOCs seen in normal Ringer’s at −50 mV. (C) Whole cell outward currents elicited at −20 and 40 mV, comparing the amplitude of the sustained Iₙo and the apparent amplitude of the transient Iₙo. (D) Normalized I-V plot of the whole cell outward currents. Region enclosed within arrows indicates the voltage range of SMOC appearance. The magnitude of Iₙo (closed circles; measured at the end of the 500 ms pulse) and the apparent magnitude of Iₙo (open squares) have been normalized to their peak values. Data is an average of eight cells.
makes up only 59.2 ± 4.6% of \(I_{\text{iso}}\); a shift in favor of the voltage-dependent component. SMOCs, similar to the \(I_{\text{iso}}\) and \(I_{\text{iso-Ca}}\), were eliminated at 50 mV in the presence of 6 mM Co\(^{2+}\)/0 mM Ca\(^{2+}\) Ringer’s, thus suggesting that they too were Ca\(^{2+}\) dependent (see next section).

**Ca\(^{2+}\) Influx through VGCCs Is Required for the Generation of SMOCs**

Reducing Ca\(^{2+}\) influx through VGCCs by the addition of inorganic blockers to normal Ringer’s (6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s), led to the generation of SMOCs at −20 mV and above, while eliminating them at hyperpolarized voltages. Representative results are shown in Fig. 3, A1 and A2 (\(V_{\text{cmd}}\) steps to −50 and 30 mV; \(n = 20\)). This is in contrast to the appearance of SMOCs in the narrow voltage range of −60 to −40 mV in normal Ringer’s. SMOCs generated in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s at −10 mV had a mean amplitude of 158.2 ± 2.6 pA, mean time to peak of 3.7 ± 0.1 ms and a mean 95% decay time of 6.2 ± 0.1 ms (average data from ~800 events in five cells). In this set of five cells used for analysis, SMOCs appeared at a frequency of 10.7 ± 0.4 Hz at 30 mV. A plot from a representative cell showing SMOC frequency versus inorganic blocker dose at 30 mV is shown in Fig. 3 B. Increasing the cobalt concentration, while maintaining 1.8 mM Ca\(^{2+}\), reduced SMOC frequency. 20 mM cobalt eliminated SMOCs. In this cell, SMOC frequency was 21 ± 0.6 Hz at 4 mM Co\(^{2+}\) and was reduced to 7.2 ± 0.6 Hz at 8 mM cobalt (\(P < 0.01\)). Average data from five cells under similar conditions gave SMOC frequencies of 23.4 ± 1.2 Hz at 4 mM Co\(^{2+}\) and 7.9 ± 0.5 Hz under 8 mM Co\(^{2+}\) (\(P < 0.01\)). Addition of 100 μM Cd\(^{2+}\), instead of Co\(^{2+}\), also led to generation of SMOCs at depolarized voltages (\(n = 15\)). These effects of inorganic blockers were fully reversible on washout.

Fig. 3 C shows representative SMOCs generated at +30 mV in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s (Fig. 3 C1) and 6 mM Co\(^{2+}\)/0 mM Ca\(^{2+}\) Ringer’s (Fig. 3 C2) solutions, respectively. The cell was exposed to the
nominally Ca\(^{2+}\)-free solution for 10 s and SMOCs were eliminated. However, these SMOCs were immediately reinstated on return to control 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s (Fig. 3 C3) \((n = 15)\). Subsequent sections show that release of intracellularly stored Ca\(^{2+}\) is involved in the SMOC generation pathway. Although prolonged (tens of minutes) exposure to Ca\(^{2+}\)-free solution can deplete the stores of Ca\(^{2+}\), it is unlikely that 10 s exposures would cause such an effect. Moreover, brief exposures to low doses of caffeine (1–3 mM) after SMOCs were eliminated in nominally Ca\(^{2+}\)-free solution could reinstate SMOC-like activity \((n = 5)\), suggesting the stores were replete under these conditions.

The dose-dependent reduction in SMOC frequency with increasing doses of inorganic blocker, and their elimination in nominal Ca\(^{2+}\)-containing solutions suggest that Ca\(^{2+}\) influx through VGCCs is involved in the SMOC generation pathway. Another possible route of Ca\(^{2+}\) influx into the cell is via receptor-operated channels (Betz, 1990; Gilbertson et al., 1991; Brorson et al., 1992; Kostyuk and Verkhratsky, 1994). In retinal third order neurons, the kainic acid (KA) receptor, a glutamate receptor subtype, has been shown to be permeable to Ca\(^{2+}\) (unpublished data; Otori et al., 1998; Taschenberger and Grantyn, 1998; Yoon et al., 1999). Addition of 50 \(\mu\)M KA to cells did not alter SMOC frequency \((n = 11)\). SMOC frequency in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s at 30 mV was 5.3 ± 0.6 Hz. Addition of 50 \(\mu\)M KA resulted in a SMOC frequency of 5.2 ± 0.7 Hz (average data from three cells, difference between frequency values is not significant at \(P < 0.05\)). Increasing the driving force for KA-induced Ca\(^{2+}\) influx by generating SMOCs at −10 mV \((n = 3)\) or −50 mV \((n = 5)\) did not result in any significant change in frequency. These observations indicate that Ca\(^{2+}\) influx through VGCCs is necessary for SMOC generation.

SMOCs generated in the presence of cobalt were more stable, gave a better signal to noise ratio, and afforded a broader dynamic range of voltage activation. Due to these experimental advantages, it was far easier to unambiguously identify and quantitate SMOCs generated in this manner, as compared with those seen within a narrow voltage range in normal Ringer’s. Therefore, the properties of SMOCs were examined in the presence of
cobalt. However, equivalent studies, when feasible, were done on SMOCs generated in normal Ringer’s. The results of the two paradigms were qualitatively similar.

SMOCs occurred randomly irrespective of whether they were generated in normal or Co²⁺-containing Ringer’s. Fig. 3 D shows an interevent interval histogram for SMOCs generated in 6 mM Co²⁺/1.8 mM Ca²⁺ Ringer’s. The exponential interevent interval distribution obtained suggests the random nature of their occurrence.

**Ca²⁺ Influx through High Voltage–activated (HVA) Ca²⁺ Channels Is Responsible for SMOC Generation**

Since VGCCs are important for SMOC generation, the type of calcium channel was examined. SMOCs recorded in normal Ringer’s are generated within a relatively hyperpolarized voltage range of −60 to −40 mV. It is possible that Ca²⁺ influx through low voltage–activated Ca²⁺ channels (T-type) might be involved. Lacking a pharmacological method of blocking this channel, a common biophysical approach is to inactivate T-type channels by holding the cells at depolarized voltages (Narahashi et al., 1987; Bean, 1989). With the holding potential at −80 mV, SMOCs generated at −50 mV in normal Ringer’s had a frequency of 15.3 ± 1.6 Hz. Maintaining the holding potential at −50 mV resulted in a SMOC frequency of 13 ± 1.4 Hz (average from four cells; difference in frequencies was not significant at *P* < 0.05). Similarly, SMOCs generated in 6 mM Co²⁺/1.8 mM Ca²⁺ by a step to −10 mV also failed to be eliminated when the holding voltage was −40 mV (*n* = 3; unpublished data). These results suggest that T-type channels are not involved in SMOC generation.

Fig. 4 A shows representative SMOC recordings obtained after a depolarization to −10 mV in 6 mM Co²⁺/1.8 mM Ca²⁺ Ringer’s in the absence (A1) and presence (A2) of 10 μM nifedipine, a blocker of L-type Ca²⁺ channels. Fig. 4 B shows that nifedipine produces...
a graded, dose-dependent reduction in SMOC frequency. In this cell, nifedipine reduced SMOC frequency from a control value of 54.6 ± 2.9 Hz to 22 ± 1.6 Hz at 10 µM (P < 0.01). However, even at doses of 60 µM, nifedipine was unable to totally block SMOC generation. Average data from seven cells in which SMOCs were recorded under 6 mM Co²⁺/1.8 mM Ca²⁺ Ringer’s at 30 mV gave control frequencies of 20 ± 1.6 Hz, but 8.6 ± 0.6 Hz in 10 µM nifedipine (P < 0.01). This effect of nifedipine was partially reversible and was qualitatively confirmed in a total of 18 cells. Addition of S (−) BayK 8644 (3 µM), an L-type Ca²⁺ channel agonist, had the opposite effect. Traces from a representative cell are shown in Fig. 4, C1 (control) and C2 (agonist), for SMOCs generated in 6 mM Co²⁺/1.8 mM Ca²⁺ Ringer’s with a voltage step to −10 mV. Control SMOC frequency in this cell was 4.5 ± 0.6 Hz, and it increased to 14.8 ± 0.7 Hz (P < 0.01) on addition of the agonist (Fig. 4 D). Average data from six cells in which SMOCs were recorded under similar conditions gave control frequencies of 7.6 ± 0.8 Hz, but 15.6 ± 1 Hz in S (−) BayK 8644 at the same dose also enhanced the frequency of SMOCs generated in normal Ringer’s at −50 mV (n = 3; unpublished data). The effect of the agonist was not reversible. The lack of reversibility of DHP agents has been noted in earlier studies and is probably due to their lipid solubility and uptake into membranes (Pang and Sperelakis, 1984; Nerbonne and Gurney, 1987). The reduction of SMOC frequency by nifedipine and enhancement by S (−) Bay K 8644 strongly suggest that L-type HVA Ca²⁺ channels play a role in SMOC generation. However, the lack of a complete block by high doses of nifedipine leaves open the question of whether this is the only subtype of HVA Ca²⁺ channels involved. Although there is a possibility that other VGCC subtypes, such as N, P, Q, and R, may participate in this process (Bean, 1989; Zhang et al., 1993; Dunlap et al., 1995), a pharmacological examination of the influence of these other Ca²⁺ channels on SMOC frequency was not performed.

**SMOCs Are K⁺ Currents: Ion Substitution Experiments**

In muscle cells, spontaneous inward Cl⁻ fluxes have also been reported, termed spontaneous transient inward currents (STICs) (Wang et al., 1992; Janssen and Sims, 1994; Henmi et al., 1996). Experiments to determine the ion selectivity of spontaneous events in retinal neurons are summarized in Fig. 5. Eₖ was varied while E_Cl was kept constant by equimolar substitution of NaCl by KCl. Fig. 5 A shows representative SMOCs with 2.5 mM [K⁺]ₑₓ, and with 25 mM [K⁺]ₑₓ at 20 mV in 6 mM Co²⁺/1.8 mM Ca²⁺ Ringer’s. The mean SMOC amplitude in this cell was 78 ± 4 pA in 2.5 mM [K⁺]ₑₓ, and 36 ± 1 pA
in 25 mM [K+]_{\text{ext}}. Mean SMOC amplitude decreased as the [K+]_{\text{ext}} increased. Fig. 5B plots mean SMOC amplitude versus log [K+]_{\text{ext}}. The data points are average values obtained from four cells in which SMOCs were generated at −10 mV by the addition of 6 mM Co^{2+}/1.8 mM Ca^{2+} Ringer’s with various K+ concentrations. SMOCs had mean amplitudes (in pA) of 169.69 ± 4.4 (2.5 mM [K+]_{\text{ext}}), 117.01 ± 2.2 (10 mM [K+]_{\text{ext}}), 89.79 ± 2.2 (17.5 mM [K+]_{\text{ext}}), 83.86 ± 4 (25 mM [K+]_{\text{ext}}), and 48.92 ± 1.8 (37.5 mM [K+]_{\text{ext}}). A linear fit provides a slope of −106.69 under these conditions; corresponding to an average conductance of 1830 pS or ~16 BK channels at the peak of the SMOC (see discussion).

SMOCs generated in normal Ringer’s at −55 mV could be made to reverse direction by increasing [K]_{\text{ext}} to 110 mM (high K+ Ringer’s; n = 4). In three out of the four cells tested, switching to high K+ Ringer’s resulted in a large holding current and an eventual obscuring of SMOCs at this negative voltage. Although the cause for this is not clear, it is most likely due to an enhancement of the resting K+ leak conductance. Moreover, the elicited sustained K+ currents showed a marked slowing of deactivation under such conditions, which additionally contributed to this effect (Swenson and Armstrong, 1981). Nevertheless, SMOCs were observed to reverse direction immediately after switching to the high K+ Ringer’s. This is shown in Fig. 5C. The arrow in Fig. 5C2 marks the point at which the solution change was started. The baseline current gradually increased and SMOCs became inward. The effects of high K+ Ringer’s were fully reversible. These ion substitution studies suggest that retinal SMOCs are primarily K+ currents.

SMOCs Are K+ Currents: Pharmacological Block

SMOCs recorded in Ca^{2+}-containing solutions were blocked completely by the K+ channel blocker, TEA (10 mM; n = 10). Representative traces showing TEA block of SMOCs generated at −10 mV in 6 mM Ca^{2+}/1.8 mM Ca^{2+} Ringer’s are shown in Fig. 6A. The TEA block was fully reversible. Increasing concentrations of TEA produced a graded reduction of SMOC amplitude at −10 mV in 6 mM Ca^{2+}/1.8 mM Ca^{2+} Ringer’s (Fig. 6B). In this cell, SMOCs had a mean amplitude of 159 ± 3 pA under control conditions and 91 ± 2 pA in the presence of 0.5 mM TEA. Average SMOC data from four cells under similar conditions gave control amplitudes of 204.9 ± 6.4 pA, and 108.4 ± 3.1 pA in 500 μM TEA (a reduction of ~47%; P < 0.01). TEA also produced a dose-dependent decline in SMOC frequency (unpublished data). This is probably due to reduction in amplitude by TEA, causing SMOCs of smaller amplitude to merge with the baseline noise and escape detection. SMOCs generated in normal Ringer’s at −50 mV were also blocked by 10 mM TEA (n = 4). SMOCs were not blocked by 5 mM 4-aminopyridine (4-AP; n = 9). Prolonged exposure (>2 min, as compared with a perfusion equilibration time with an upper limit of 10 s) to 4-AP resulted in a gradual, irreversible decline in SMOC frequency and subsequent elimination of SMOCs. This was not reversible on returning to control. However, SMOCs could be reinstated after pulsing to depolarized levels in normal Ringer’s, a process that presumably permits Ca^{2+} influx into the cell and replenishes stores. This effect of prolonged 4-AP application may be nonspecific. It is reminiscent of a gradual rundown/depletion phenomena, possibly arising from effects of 4-AP on other molecules, such as the Ca^{2+} ATPases (Ishida and Honda, 1993). The significant blockade of SMOCs by submillimolar amounts of TEA further substantiates that SMOCs are currents generated via fluxes through K+ channels.

SMOCs Are Currents through Large Conductance Ca^{2+}-activated K+ Channels (BK Channels)

Fig. 6C shows that SMOCs generated in 6 mM Ca^{2+}/1.8 mM Ca^{2+} Ringer’s can be eliminated by the addition of a blocker of BK channels, 100 nM iberiotoxin (n = 5, V_{cmd} = −10 mV). The onset of iberiotoxin block is relatively slow (2 min) and like TEA, there was a graded reduction in SMOC amplitude and frequency (unpublished data). The block by iberiotoxin was not reversible on switching back to toxin-free control solution.

Since intracellularly stored Ca^{2+} is involved in the SMOC generation pathway, care was taken to ensure that the slow time course and irreversibility of iberiotoxin block was not mistaken for rundown/depletion. Control recordings for 2 min did not show a significant change in SMOC frequency or amplitude. However, addition of 100 nM iberiotoxin led to a total block of SMOCs in the subsequent 2 min. Moreover, contrary to what would be expected for depletion-induced phenomena, refilling of stores by pulsing to depolarized levels in normal Ringer’s failed to reinstate SMOCs. This suggested a persistence of iberiotoxin block independent of depletion of the Ca^{2+} store.

Apamin (100 nM), a blocker of small conductance Ca^{2+}-activated K+ channels (SK channels), did not eliminate SMOCs generated at depolarized levels in 6 mM Ca^{2+}/1.8 mM Ca^{2+} Ringer’s nor affect their characteristics (n = 7; unpublished data). SMOCs generated in normal Ringer’s at −50 mV were blocked by 100 nM iberiotoxin, but not by 100 nM apamin (n = 8; unpublished data). These results suggest that SMOCs are K+ fluxes through the BK subtype of Kca channels.
SMOCs in Retinal Neurons

CICR Mediates SMOC Generation

Although the earlier sections showed that Ca\(^{2+}\) influx through HVA Ca\(^{2+}\) channels plays a role in the generation of SMOCs, there are reports in other neuronal preparations of SMOCs being activated by CICR triggered by Ca\(^{2+}\) influx. Ca\(^{2+}\) release from internal stores also mediates the generation of STOCs in muscle cells (see introduction). Moreover, SMOCs were induced under conditions of inorganic Ca\(^{2+}\) channel blockade, where Ca\(^{2+}\) influx was severely reduced. Under such conditions an internal amplification mechanism is likely to exist. To test this possibility, we internally dialyzed neurons with ryanodine, a plant alkaloid that blocks RyRs at high doses (Zucchi and Ronca-Testoni, 1997). Internal dialysis of ryanodine (200 \(\mu\)M) abolished SMOCs generated either in normal Ringer’s or in Co\(^{2+}\)-containing solutions (A1). 100 pA (A2), 63 pA (A3), and 155 pA (A4). 0.5 mM TEA (A2). Addition of 10 mM TEA abolished SMOCs (A3). Control SMOCs were immediately recorded, before sufficient dialysis took place. Fig. 7 A1 shows an example in which control SMOCs were recorded 17 s after achieving the whole cell configuration. These SMOCs were recorded in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s at 30 mV with 200 \(\mu\)M ryanodine added to the internal pipette solution. Dialysis of the neuron for a further 35 s led to the total elimination of SMOCs (Fig. 7 A2). The methylxanthine, caffeine, is known to sensitize the ryanodine receptor Ca\(^{2+}\) release channel, such that CICR occurs in response to much lower levels of [Ca\(^{2+}\)]. Even resting levels of [Ca\(^{2+}\)] are sufficient to cause CICR in the presence of caffeine (Verkhratsky and Shmigol, 1996). Thus, high doses of caffeine (10 mM) deplete stored Ca\(^{2+}\) within cells. Consequently, any process dependent on CICR for its activation should be eliminated and not reappear until these stores are replenished. Consistent with this expectation, it was found that 10 mM caffeine eliminated SMOCs generated either in normal Ringer’s or in the presence of inorganic blockers (n = 17). Fig. 7 B1 shows an example in which SMOCs were generated at −10 mV in the presence of 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s. Exposure to 10 mM

Figure 6. Effects of K\(^+\) channel blockers on SMOCs. (A) SMOCs were elicited with voltage steps to −10 mV in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s. Control SMOCs shown in A1 were reduced in amplitude by 0.5 mM TEA (A2). Addition of 10 mM TEA abolished SMOCs (A3). The blockade by TEA was fully reversible on washout of the drug (A4). SMOCs were superimposed on baseline currents of 151 pA (A1), 108 pA (A2), 63 pA (A3), and 155 pA (A4). (B) Effect of increasing doses of TEA on peak SMOC amplitudes. Data obtained from the same cell and values at each dose are significantly lower than control (asterisk indicates P < 0.01). (C) Control SMOCs generated at −10 mV in 6 mM Co\(^{2+}\)/1.8 mM Ca\(^{2+}\) Ringer’s to which 0.1% wt/vol BSA was added (C1). Addition of 100 nM iberiotoxin eliminated SMOCs (C2).
Caffeine completely eliminated SMOCs (Fig. 7 B2). Caffeine is known to have some other nonspecific effects such as blockade of K+ channels (Reiser et al., 1996). Although this possibility cannot be totally discounted, it is likely that store depletion accounts for SMOC elimination for the following reasons: (a) the baseline K+ current at -10 mV was not affected by caffeine, whereas SMOCs were eliminated, and (b) it is expected that any block should be relieved on washout of the blocking agent. In contrast, store depletion–induced effects can recover only after replenishment of the stores. Consistent with this, SMOCs did not recover rapidly after washout of caffeine. They could be reinstated to their original frequencies (Fig. 7 B3) only after the cell was switched to normal Ringer’s and pulsed repeatedly to 30 mV to replenish the internal calcium stores. This necessity for pulsing to depolarized levels to recover the original SMOC activity after caffeine treatment was most apparent in three cells in which SMOCs were generated in normal Ringer’s at -50 mV. Based on evidence in other neurons, it is proposed that pulsing to depolarized levels in normal Ringer’s activated the HVA Ca2+ channels, resulting in a substantial Ca2+ influx into the cell, which eventually led to store replenishment (Brorson et al., 1991; Friel and Tsien, 1992; Shmigol et al., 1994; Garaschuk et al., 1997). In contrast to other neurons displaying SMOCs, we did not observe that high doses of caffeine induced an outward current (Merriam et al., 1999; Arima et al., 2001).

Although prolonged exposure to high doses of caffeine causes store depletion, caffeine should initially be able to stimulate CICR and augment any process dependent on it. This was verified by exposing cells transiently to low doses of caffeine (1–3 mM). In a representative cell, control SMOCs were generated in 6 mM Co2+/0.9 mM Ca2+ Ringer’s at -10 mV (Fig. 7 C1). Exposure to 3 mM caffeine increased SMOC frequency in four cells (Fig. 7 C2).
SMOC activity was eliminated by BAPTA but not by EGTA

As expected for any \([\text{Ca}^{2+}]_i\)-dependent process, intracellular \(\text{Ca}^{2+}\) chelators have been shown to eliminate both STOCs and SMOCs (Benham and Bolton, 1986; Bychkov et al., 1997; Merriam et al., 1999). Since CICR was shown to be involved in the SMOC generation pathway in retinal neurons, the effect of internal \(\text{Ca}^{2+}\) chelators was tested. The affinities of both BAPTA and EGTA for \(\text{Ca}^{2+}\) are similar, although BAPTA is known to have an association rate that is \(\sim 150\) times greater as compared with EGTA (Naraghi, 1997). Thus, contrasting the effects of these two chelators would allow distinction between fast and slow \(\text{Ca}^{2+}\)-stimulated events over short distances. BAPTA eliminated SMOCs generated either in normal Ringer’s or \(\text{Co}^{2+}\)-containing solutions. An example is shown in Fig. 8 A, in which SMOCs were recorded 30 s after achieving the whole cell configuration at 30 mV in 6 mM \(\text{Co}^{2+}/1.8\) mM \(\text{Ca}^{2+}\) Ringer’s. The recording pipette contained 10 mM BAPTA (A) or 10 mM EGTA (B). (A) Control SMOCs shown in A1 were recorded 30 s after achieving the whole cell configuration. Dialysis for a further 46 s led to elimination of SMOC activity (A2). (B) Control SMOCs shown in B1 were recorded 2 min after achieving the whole cell configuration. Dialysis of the neuron for a further 5 min had no effect on SMOC activity (B2). The sag in the traces is presumably due to inactivation of voltage-dependent potassium channels. SMOCs in A were superimposed on a baseline current that peaked at 1295 pA and decayed to 900 pA at the end of the 500 ms pulse, whereas those in B were superimposed on a current peaking at 1540 pA and decayed to 1155 pA at the end of 500 ms.
taneous miniature excitatory postsynaptic currents (sEPSCs).

In these amacrine cells, depolarizing shifts in voltage elicit a successive series of outward currents: SMOCs, $I_{sO-Ca}$, and $I_{sO-V}$. The $I_{sO-Ca}$ can be further decomposed into $I_{sO-Ca}$ and a purely voltage-dependent $I_{sO-V}$. Except for the $I_{sO-Ca}$, the other three outward currents are Ca$^{2+}$-sensitive. It is shown in the following paper that iberiotoxin not only eliminates SMOCs and the $I_{sO-V}$, it also reduces the $I_{sO-Ca}$. However, depletion of the internal stores with caffeine eliminates SMOCs and the $I_{sO-V}$, but does not affect the $I_{sO-Ca}$ (Mitra and Slaughter, 2002, this issue). Thus, all three outward currents have components generated by BK channels, the distinction being that $I_{sO-Ca}$ is not activated by CICR.

**Properties of the Sustained Outward Current**

The $I_{sO}$ appeared at $-30 \text{ mV}$ and increased linearly with depolarization. It consisted of calcium-dependent ($I_{sO-Ca}$) and calcium-independent ($I_{sO-V}$) components. $I_{sO-V}$ activated and then increased linearly with depolarization. $I_{sO-Ca}$ activated at slightly more negative potentials, but showed a decline at positive voltages. When the contribution of $I_{sO-Ca}$ and $I_{sO-V}$ to total $I_{sO}$ is considered, the percentage of $I_{sO-V}$ increased with depolarizations and that of the $I_{sO-Ca}$ decreased. This decline of the $I_{sO-Ca}$ fraction may be due to the decline in Ca$^{2+}$ influx with depolarization. It is known that K$_{Ca}$ channels (BK subtype) are activated by concerted influences of both [Ca$^{2+}$] and voltage. Their sensitivity to Ca$^{2+}$ increases with membrane depolarization (Barrett et al., 1982; Vergara et al., 1998). Therefore, the decline of the $I_{sO-Ca}$ fraction with depolarization reflects the interaction between the activation-favoring effects of depolarized potentials and the activation-reducing effects of lowered Ca$^{2+}$ influx at those voltages.

**SMOC Properties**

Our results indicate that SMOCs are transient K$^{+}$ fluxes through plasmalemmal BK channels and originate from Ca$^{2+}$ influx through VGCCs; this influx being amplified by CICR from caffeine- and ryanodine-sensitive intracellular stores. Earlier studies in smooth muscle reported STOCs, which are similar to SMOCs (Benham and Bolton, 1986; Bolton and Imaizumi, 1996). Although similar, neuronal SMOCs are more transient than smooth muscle STOCs (Merriam et al., 1999). Retinal SMOCs show an interevent interval distribution that is exponential, characteristic of stochastic events (Fatt and Katz, 1952; Hubbard et al., 1969). This is in agreement with reports of randomly occurring SMOCs in cultured bullfrog neurons (Satin and Adams, 1987).

In smooth muscle, spontaneous activation of potassium or chloride channels produces currents of opposite polarity. Thus, smooth muscle exhibits STOCs when discrete release of Ca$^{2+}$ from internal stores activates potassium channels, and STICs when the same processes activate chloride channels (Wang et al., 1992; Janssen and Sims, 1994; Henmi et al., 1996). But in neurons, either conductance will produce an outward current. Retinal SMOCs are K$^{+}$ currents, since their mean amplitude varied with the [K$^{+}$]$_{st}$ in a Nernstian manner. Since they are blocked by iberiotoxin and insensitive to apamin, they likely represent openings of clusters of BK channels (McManus, 1991; Kaczorowski et al., 1996). Extracellular TEA, which decreases the amplitude of single channel currents through BK channels in a concentration-dependent manner (Saunders and Farley, 1992), reduced retinal SMOC amplitudes in a dose-dependent manner, eliminating them at 10 mM. These findings concur with published data on both STOCs and SMOCs in other cell types, which show them to be coordinated openings of $K_{Ca}$ channels of the BK subtype (Bolton and Imaizumi, 1996; Merriam et al., 1999). However, small conductance $K_{Ca}$ (SK subtype) channels can also generate SMOCs, as shown in rat Meynert neurons (Arima et al., 2001).

Retinal SMOCs generated at $-50 \text{ mV}$ in normal Ringer’s displayed a mean amplitude of $33 \pm 0.5 \text{ pA}$. BK channels have been reported to have conductances in the range of 115–118 pS in third order neurons in mammalian retina (Lipton and Tauck, 1987; Wang et al., 1998). Assuming a similar conductance in amphibian neurons, it is estimated that retinal SMOCs generated in normal Ringer’s represent an average opening of $\sim$6 BK channels at their peak. The maximum SMOC height seen in normal Ringer’s at this voltage was 92 pA, which would represent the opening of $\sim$18 BK channels. SMOCs generated in 6 mM Co$^{2+}$/1.8 mM Ca$^{2+}$ Ringer’s at $-10 \text{ mV}$ had a mean amplitude of $158 \pm 3 \text{ pA}$ corresponding to $\sim$16 BK channels. The slope of the fitted data shown in Fig. 5 B gives a similar estimate. Fletcher and Chiappinelli (1992) examined spontaneous miniature hyperpolarizations (SMHs; the voltage equivalent of SMOCs) in chick ciliary ganglion neurons and reported $\sim$15–60 channels in each SMH, whereas Scornik et al. (2001) in mudpuppy cardiac neurons arrived at a value of 18–23 channels in the average SMOC. SMOCs in retinal neurons are similar to these reports, rather than bullfrog sympathetic neurons wherein each SMOC was reported to constitute activation of 10–5,000 channels (Satin and Adams, 1987). In spite of the small number of channels, single channel currents were not observed. Perhaps the recording bandwidth led to the filtering of such single steps.

**Voltage-gated Calcium Channels Initiate SMOCs**

The Ca$^{2+}$ ions necessary for the activation of $K_{Ca}$ channels can be provided either via extracellular influx or release from intracellular stores (Zorumski et al., 1989;...
Wigdirda and Dryer, 1994; Davies et al., 1996; Marrion and Tavali, 1998; Hurley et al., 1999; Imaizumi et al., 1999). Satin and Adams (1987), in their study of SMOCs in bullfrog sympathetic neurons, reported that Ca\textsuperscript{2+} influx through surface membrane Ca\textsuperscript{2+} channels was not essential for SMOC generation. They suggested that neuronal depolarization directly coupled to Ca\textsuperscript{2+} release from internal stores. Their conclusions were based on the finding that 100 \mu M Cd\textsuperscript{2+} did not block SMOCs. Fletcher and Chiappinelli (1992) found a similar insensitivity to external Ca\textsuperscript{2+} in chick ciliary ganglion neurons. Arima et al. (2001) suggest that Ca\textsuperscript{2+} influx contributes to, but is not essential, for SMOC generation in rat Meynert neurons. However, other investigators found Ca\textsuperscript{2+} influx was required for SMOC generation (Mathers and Barker, 1981, 1984; Merriam et al., 1999). Retinal SMOCs were instantly eliminated when cells were exposed to nominal [Ca\textsuperscript{2+}] containing solutions, and immediately recovered when bath Ca\textsuperscript{2+} was reinstated.

Retinal SMOCs were initiated by Ca\textsuperscript{2+} influx through plasmalemmal VGCCs. SMOCs are seen in normal Ringer's at membrane potentials between -60 to -40 mV. Addition of inorganic VGCC blockers such as Co\textsuperscript{2+} or Cd\textsuperscript{2+} eliminated SMOCs at these hyperpolarized levels, although they reappeared at more depolarized voltages, at or above -20 mV. It is generally thought that these divalent cations are VGCC pore blockers (Lansman et al., 1986; Vinegar et al., 1991). Therefore, depolarization would tend to push the blocker out of the channel and thus relieve block (Woodhull, 1973; Fukushima and Hagiwara, 1985; Thevenod and Jones, 1992; Carbone et al., 1997; Wakamori et al., 1998). Increasing the dose of the inorganic blocker resulted in a graded reduction in retinal SMOC frequency. Stimulating non-VGCC-mediated Ca\textsuperscript{2+} influx by activation of kainic acid receptors did not affect SMOC frequency. These observations suggested that Ca\textsuperscript{2+} influx through VGCCs is necessary for SMOC generation.

DHPs are a group of Ca\textsuperscript{2+} channel ligands that are thought to be selective for the L-type HVA Ca\textsuperscript{2+} channel (Bean, 1984; Nowycky et al., 1985; Fox et al., 1987; Rane et al., 1987). L-type HVA Ca\textsuperscript{2+} channels are known to be in close proximity to the RyRs in cardiac muscle, and it is presumably this anatomical arrangement that enables the occurrence of discrete Ca\textsuperscript{2+} release events called "sparks" (Carl et al., 1995; Sun et al., 1995). In smooth muscle cells, sparks have been implicated as the underlying event giving rise to STOCs (Nelson et al., 1995; Mironneau et al., 1996; Perez et al., 1999; ZhuGe et al., 1999). A similar anatomical relationship may also exist in smooth muscle cells (Jaggar et al., 1998). Our experiments revealed the involvement of L-type HVA Ca\textsuperscript{2+} channels in retinal SMOC generation. The DHP blocker, nifedipine, reduced SMOC frequency in a dose-dependent manner and the agonist S (-) BayK 8644 increased it. Although nifedipine reduced SMOC frequency, it never completely eliminated SMOCs, even at concentrations up to 60 \mu M at relatively depolarized voltage of 30 mV. This result may be interpreted in two ways. One is that other subtypes of HVA calcium channel, such as N, P, Q, and R, may be involved (Bean, 1989; Zhang et al., 1993; Dunlap et al., 1995), and that the system does not show any specificity as regards the type of HVA Ca\textsuperscript{2+} channel that generates SMOCs. This was the conclusion arrived at by Merriam et al. (1999) in their study of SMOCs in mudpuppy parasympathetic cardiac neurons. The involvement of other subtypes of HVA Ca\textsuperscript{2+} channels was not examined in this present study. The alternative explanation is that the L-type VGCCs are not totally blocked, even with high doses of nifedipine, and the consequent high amplification of the trigger Ca\textsuperscript{2+} signal by CICR leads to a SMOC. Blockade by DHP antagonists is complex since cell voltage affects it. These blockers have a higher affinity for the inactivated state of the channel, the population of which increases as the cell is depolarized (Bean, 1984; Sanguinetti and Kass, 1984). It may be that this voltage dependence of nifedipine block does not allow a 100% block even at depolarized voltages, permitting a stochastic unblock of a few Ca\textsuperscript{2+} channels. It has been shown in heart muscle that influx through one Ca\textsuperscript{2+} channel is enough to activate CICR (Santana et al., 1996). This would be all the more likely if the intracellular stores are filled with Ca\textsuperscript{2+}, since it has been shown that store filling increases the sensitivity of the stores to generate CICR, whereas store depletion has the opposite effect (Friel and Tsien, 1992; Jaffe and Brown, 1994; Shmigol et al., 1996; Hernandez-Cruz et al., 1997; Usachev and Thayer, 1997). Although demonstrating the involvement of L-type Ca\textsuperscript{2+} channels in the SMOC generation pathway, this study does not eliminate the involvement of other HVA types.

**Calcium-induced Calcium Release Produces SMOCs**

Ca\textsuperscript{2+} influx through VGCCs can either directly activate the K\textsubscript{Ca} channels responsible for SMOCs, or trigger CICR (Sah and McLachlan, 1991; Sah, 1992; Jobling et al., 1993; Berridge, 1998). CICR-triggered via Ca\textsuperscript{2+} influx through VGCCs is the accepted mechanism of Ca\textsuperscript{2+} spark generation in cardiac muscle (Imaizumi et al., 1999). In neurons, CICR has been shown to be an intermediate step for SMOC generation (Mathers and Barker, 1981, 1984; Merriam et al., 1999). In retinal neurons, the effects of both caffeine and ryanodine indicate that CICR is involved in SMOC generation. Low concentrations of ryanodine (<10 \mu M) lock the release channel in a subconductance state, which is 40–60% of the normal conductance. Higher concentrations (>100 \mu M) of ryanodine block the release channel in a use-dependent manner (Sutko and Airey, 1996;
Caffeine, on the other hand, increases the sensitivity of the channel to trigger Ca$^{2+}$ release, causing significant release even at resting levels of [Ca$^{2+}$], (Verkhratsky and Shmigol, 1996). It increases the open probability of the release channel by generating more frequent openings, whereas the open mean time and conductance is not altered (Rousseau et al., 1988; Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990; Hernandez-Cruz et al., 1995). High doses of caffeine (10 mM) deplete the internal Ca$^{2+}$ stores and thus eliminate CICR (Friel and Tsien, 1992). Low doses of caffeine (1–3 mM) have been shown to augment CICR-dependent process by causing additional Ca$^{2+}$ release in response to the trigger signal. Internal dialysis with 200 μM ryanodine completely eliminated retinal SMOCs. Exposure to 10 mM caffeine eliminated retinal SMOCs, which recovered only after store refilling. Caffeine at low doses (1–3 mM) increased SMOC frequency. Similar effects of low doses of caffeine have been observed on both STOCs and SMOCs (Bolton and Imaizumi, 1996; Merriam et al., 1999; Arima et al., 2001). Retinal SMOCs induced by 1–3 mM caffeine showed a prolonged decay time. Merriam et al. (1999) made a similar observation in mudpuppy parasympathetic neurons, suggesting this reflected caffeine’s ability to cause the release channel to open more frequently. As a consequence, there are multiple activations of the BK channels in the vicinity of the release channel, slowing the rate of decay.

Internal BAPTA eliminated SMOC activity, whereas EGTA did not. BAPTA and EGTA have similar affinities for Ca$^{2+}$, but the former has faster association kinetics (Naraghi, 1997). Consequently, BAPTA can eliminate rises of [Ca$^{2+}$], within more restricted microdomains (Deisseroth et al., 1996; Sham, 1997; Neher, 1998). This differential effect of the two buffers suggests that the [Ca$^{2+}$], rise responsible for activating the BK channels is a highly localized event. A similar observation was reported by Merriam et al. (1999) in mudpuppy cardiac neurons. Such localized [Ca$^{2+}$], elevations, “Ca$^{2+}$ sparks,” have been noted in cardiac and smooth muscle (Imaiizumi et al., 1999). Based on these findings and the mechanism known to exist in smooth muscle, it would be a reasonable conjecture that a “Ca$^{2+}$ spark” underlies SMOC generation in retinal neurons.

In summary, our experiments show that, in a subset of retinal amacrine cells, Ca$^{2+}$ influx through L-type HVA Ca$^{2+}$ channels triggers CICR from caffeine and ryanodine-sensitive stores. This amplified Ca$^{2+}$ signal subsequently activates clusters of BK channels leading to outward K$^+$ current fluxes which appear as SMOCs. However, there is a complex relationship between Ca$^{2+}$ influx and SMOC appearance. Although Ca$^{2+}$ influx was found to be a prerequisite for SMOC generation, reducing Ca$^{2+}$ influx caused SMOCs to appear at depolarized voltages while eliminating them at hyperpolarized levels. This would suggest that large amounts of Ca$^{2+}$ influx probably has a negative modulatory effect on SMOCs, since the open probability of VGCCs is usually higher at depolarized voltages. This is addressed in the subsequent paper, which characterizes the biophysical properties of SMOCs, including their Ca$^{2+}$ dependence, and thus provides an explanation for their disappearance at depolarized voltage levels in normal Ringer’s. These findings suggest that SMOCs putatively serve to suppress spontaneous excitatory synaptic events within the retinal network (Mitra and Slaughter, 2002, this issue).

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