1. Projektbezeichnung:

$Ca_v1.2$ L-type Ca^{2+} channels control release of Ca^{2+} sparks in arterial smooth muscle

2. Kurztitel:

Cav1.2 and Ca^{2+} sparks

Antragsteller:

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3. Zusammenfassung

Ca^{2+} sparks are released by opening ryanodine receptors (RyRs) in the sarcoplasmic reticulum (SR). In arterial smooth muscle cells (SMC), Ca^{2+} sparks stimulate nearby Ca^{2+} activated K^+ (BK) channels, causing spontaneous transient outward currents that hyperpolarize the membrane potential and oppose myogenic vasoconstriction. It is well known that intimate association between the trigger $Ca_v1.x$ channel and target RyR is crucial for the control of Ca^{2+} sparks in heart and skeletal muscle. However, the equivalent coupling process in SMC is not clear to date. We will use smooth muscle-specific inactivation of the $Ca_v1.2$ channel and test the hypotheses that (1) genetic inactivation of $Ca_v1.2$ channels reduces Ca^{2+} spark frequency and amplitude; (2) these effects are associated with lower global cytosolic Ca^{2+} levels and reduced SR Ca^{2+} load; and, (3) are completely reversed by elevating cytosolic Ca^{2+} levels. We will show that $Ca_v1.2$ channels activate RyRs to release Ca^{2+} sparks through control of the global cytosolic $[Ca^{2+}]$.

4. Stand der Forschung und eigene Vorarbeiten

Several diverse cellular functions such as cell contraction, fertilization, proliferation, secretion, and information processing are controlled by the ability of cells to generate highly sophisticated Ca^{2+} signals. These signals are characterized by a high degree of spatial-temporal complexity (Berridge et al., 2003). In arterial smooth muscle, increases in the global cytosolic Ca^{2+} concentration stimulate contraction by activating myosin light chain kinase. The increase is mainly due to Ca^{2+} ions entering arterial smooth muscle cells (SMC) through voltage-dependent L-type $Ca_v1.2$ Ca^{2+} channels. These activating Ca^{2+} signals, although not reaching very high $[Ca^{2+}]_i$, (in the range of 100-300 nM) concentrations, are distributed throughout the entire cell (global) and last long enough to permit Ca^{2+} ions to regulate vascular contraction (Nelson et al., 1990).

More recently, local $[Ca^{2+}]_i$ transients (Ca^{2+} sparks) have been visualized in heart, skeletal muscle and SMC (Nelson et al., 1995). Ca^{2+} sparks are caused by opening of ryanodine-sensitive Ca^{2+} release channels (RyR) localized in the sarcoplasmic reticulum (SR). The triggering process of Ca^{2+} sparks in heart and skeletal muscle is well characterized: sparks are generated by depolarization of the plasma membrane because voltage-gated sarcolemmal L-type Ca^{2+} channels activate the RyR, either by close coupling between the channels and RyRs (i.e., *via* RyR sensing of the local elevation of $[Ca^{2+}]_i$ in a Ca^{2+} microdomain near the $Ca_v1.2$ channel in the T-tubular membrane (Cheng et al., 1993; Gollasch et al., 2000; Guatimosim et al., 2002; Wang et al., 2004; Wehrens et al., 2004)) or by direct protein-protein interaction (Klein et al., 1996; Rios and Brum, 2002; Schneider and Ward, 2002; Shirokova et al., 1998; Stern et al., 1997; Tsugorka et al., 1995), respectively. In contrast, it is not clear to date whether or how local communication by Ca^{2+} from L-type calcium channels activates the RyR in SMC.

What is the function of Ca^{2+} sparks in SMC? A single spark is capable of producing a very high (10-100 µM) local (~ 1% of the cell volume) increase in $[Ca^{2+}]_i$ (Furstenau et al.,

2000; Perez et al., 1999), while increasing the global $[Ca^{2+}]_i$ only by < 2 nM (Jaggar et al., 2000; Nelson et al., 1995). Ca^{2+} sparks occur in close proximity to the cell membrane, where every Ca^{2+} spark activates a cluster of Ca^{2+} activated K^+ (BK) channels (Perez et al., 1999). Consistently, we demonstrated that STOCs disappeared by genetic ablation of BK channel alpha subunits (Sausbier et al, Circulation, in press) or accessory BKß1 subunits (Pluger et al., 2000). BK channels have a 10^4-fold increase in their open probability in the presence of high $[Ca^{2+}]_i$ during a Ca^{2+} spark (Perez et al., 1999), but are otherwise not responsive to the much lower global $[Ca^{2+}]_i$ (i.e., 100-300 nM) (Gollasch et al., 1998). BK currents caused by Ca^{2+} sparks were originally termed «spontaneous transient outward currents» or STOCs (Benham and Bolton, 1986). SMC STOCs cause a global hyperpolarization of the cell membrane thus closing $Ca_v1.2$ channels. Therefore, they may serve as valuable feed back mechanism to increased global $[Ca^{2+}]_i$ and arterial myogenic constriction (Gollasch et al., 1998; Knot et al., 1998; Lohn et al., 2001b; Nelson et al., 1995).

Multiple Ca^{2+} influx and release pathways are co-expressed in arterial smooth muscle cells (Bolton et al., 2002); however, whether they serve specialized functions or simply all contribute to Ca^{2+} spark activity is not known. We recently demonstrated that raloxifene blocks L-type $Ca_v1.2$ channels (Tsang et al., 2004). The L-type Ca^{2+} channel blocker diltiazem inhibited Ca^{2+} sparks in intact cerebral arteries (Jaggar et al., 1998), while the L-type activator BayK 8644 increased the frequency of Ca^{2+} sparks in smooth muscle cells of cerebral arteries (Gollasch et al., 2000; Nelson et al., 1995). Therefore, we will test the hypothesis that $Ca_v1.2$ channels contribute to Ca^{2+} spark formation in arterial vascular SMC using smooth muscle-specific inactivation of the $Ca_v1.2$ channel gene in mice (SMAKO) (Moosmang et al., 2003) and pharmacological drugs, such as dihydropyridines and raloxifene. We want to provide evidence that $Ca_v1.2$ channels are essential for maintaining specific spatial-temporal characteristics of Ca^{2+} sparks in arterial SMC. We want to show that $Ca_v1.2$ channels modulate Ca^{2+} sparks through an increase in the global cytosolic $[Ca^{2+}]$.

5.1. Bisherige bzw. beantragte Förderung des Projektes bzw. von themenverwandten Teilprojekten im Rahmen der haushaltsfinanzierten Forschungsförderung

keine

5.2. Drittmittelperspektive

angestrebte nach Förderung: DFG

vorgesehener Termin: 10/05

6. Ziele

It is well known that intimate association between the trigger $Ca_v1.x$ channel and target RyR is crucial for the control of Ca^{2+} sparks in heart and skeletal muscle. However, the equivalent coupling process in arterial smooth muscle cells (SMC) is not clear to date. We will use smooth muscle-specific inactivation of the $Ca_v1.2$ channel and test the hypotheses that (1) genetic inactivation of $Ca_v1.2$ channels reduces Ca^{2+} spark frequency and amplitude; (2) these effects are associated with lower global cytosolic Ca^{2+} levels and reduced SR Ca^{2+} load; and, (3) are completely reversed by elevating cytosolic Ca^{2+} levels. We will show that $Ca_v1.2$ channels activate RyRs to release Ca^{2+} sparks through control of the global cytosolic $[Ca^{2+}]$. This calcium-induced calcium release process will differ substantially from cardiac and skeletal muscle and, thus, will represent the third known mechanism of coupling between RyR and L-type Ca^{2+} channels realized in eukaryotic cells.

7. Arbeitsprogramm

SMC will be isolated from tibial and cerebral arteries and Ca^{2+} sparks will be recorded in fluo-3-loaded single SMC using confocal laser scanning microscopy. The techniques of confocal

line-scan recordings of Ca^{2+} sparks has been established for tibial artery SMC isolated from control and SMAKO mice (Sausbier et al.; *Circulation* in press). Ca^{2+} sparks will be observed in close proximity to the cell surface in both control and SMAKO cells. The following parameters of spark will be measured: mean amplitude, a mean rise time, decay half-life and a width at half-maximal amplitude, the frequency of Ca^{2+} sparks.

Simultaneous optical whole cell and electrical measurements in isolated SMC indicate that virtually all Ca^{2+} sparks cause STOCs in arterial myocytes (Perez et al., 1999). These measurements also point out that Ca^{2+} sparks with an amplitude of ~400 nM increase the open probability of BK channels ~10^6-fold, which is consistent with subsarcolemmal activator [Ca^{2+}] in the order of 10-100 µM (Furstenau et al., 2000; Perez et al., 1999; Zhuge et al., 2002). We will record STOCs in tibial artery SMC from both control and SMAKO by means of the perforated patch-clamp technique. These results will indicate whether L-type $Ca_v1.2$ channels are involved in the activation of RyRs to release Ca^{2+} sparks. A reduced STOC amplitude in SMAKO cells would be consistent with reduced Ca^{2+} spark amplitude, which results in lower subsarcolemmal activator [Ca^{2+}] (<10-100 µM) to activate BK channels.

To study the mechanism by which $Ca_v1.2$ channel currents activate RyRs to release Ca^{2+} sparks, we will modulate the open probability of $Ca_v1.2$ channels by dihydropyridines (Bay K 9644, nimodipine) and the $Ca_v1.2$ channel blocker raloxifene. Ca^{2+} sparks will be measured between 30 and 90 min after application of the drugs. Consistent with the view that $Ca_v1.2$ channels activate RyRs to release Ca^{2+} sparks, we expect that nimodipine (1 µM) and raloxifene (1 µM) will reduce the frequency of Ca^{2+} sparks in control cells depolarized by 30 mM KCl. In contrast, we expect that BayK 8644 (1 µM), nimodipine (1 µM), and 30 mM KCl will not affect on the Ca^{2+} spark frequency in SMAKO cells. Furthermore, we expect that nimodipine (1 µM) will not affect the Ca^{2+} spark frequency in SMAKO cells incubated in 30 mM KCl-containing bath solution. These data will be consistent with the idea that L-type

$Ca_v1.2$ channels play a major role in the generation of Ca^{2+} sparks and that sparks can be modulated by long-term application of dihydropyridines.

We next will measure STOCs in the absence and presence of dihydropyridines, raloxifene and Cd^{2+}. The effects of the drugs on STOCs will be analyzed between 1 and 15 min after drug application in tibial SMCs. Similar experiments will be performed in cerebral artery SMC.

As a result, we will hypothesize that, rather than sensing the local elevation of $[Ca^{2+}]_i$ in the microdomain near the pore of the $Ca_v1.2$ channel, smooth muscle RyRs are not sensitive to the opening of individual $Ca_v1.2$ channels, but require a global rise in cytosolic $[Ca^{2+}]$. To test this hypothesis, we will first determine whether Ca^{2+} influx is required for initiation of Ca^{2+} sparks. After removal of external Ca^{2+} the frequency of Ca^{2+} sparks will be studied after 15 min in Ca^{2+}-free solution, similar to the effects of nimodipine and Cd^{2+} (see also (Bonev et al., 1997)). As a further test, we will lower the global cytosolic $[Ca^{2+}]_i$ using 2-APB, which is known to inhibit Ca^{2+} release into the cytosol *via* IP_3 receptors (Peppiatt et al., 2003; Zima and Blatter, 2004) and to block store-operated Ca^{2+} influx (Iwasaki et al., 2001; Peppiatt et al., 2003) though non-selective, Ca^{2+} permeable, cation TRPC channels (Clapham et al., 2001; Iwasaki et al., 2001; van Rossum et al., 2000). The effects of 2-APB will be studied in the presence and absence of external Ca^{2+}. These observations will clarify whether initiation of Ca^{2+} spark release can be regulated by changes of the global $[Ca^{2+}]_i$ and the filling state of SR Ca^{2+} stores, independent of the source of Ca^{2+} (influx or release).

To demonstrate that the global resting cytosolic $[Ca^{2+}]_i$ ($[Ca^{2+}]_r$) is reduced in SMAKO cells, we will measure $[Ca^{2+}]_r$ directly. To clarify the role of $Ca_v1.2$ for stored calcium content and store-refilling, we will examine the effects of caffeine in SMAKO and control cells. These results should answer the question whether a population of $Ca_v1.2$ channels is active at resting membrane potentials and whether steady-state Ca^{2+} influx though $Ca_v1.2$ channels tightly controls the global $[Ca^{2+}]_i$ or not. We expect that caffeine (10 mM) will

evoke smaller Ca^{2+} transients in SMAKO cells, as compared to control cells, indicating that ryanodine-sensitive stores are depleted in SMAKO cells. To further characterize the disrupted Ca^{2+} uptake into ryanodine-sensitive stores in SMAKO mice, we will use a multi-pulse application protocol. We expect that 8 min after a conditioning caffeine pulse, subsequent applications of caffeine (10 mM) will induce global $[Ca^{2+}]_i$ transients only in control cells, but not in SMAKO SMC. We expect that caffeine will not induce $[Ca^{2+}]_i$ elevations at earlier time points in both cell types. Given that the recovery of the Ca^{2+} transient should be blocked in SMAKO SMC, we expect that refilling of ryanodine-sensitive Ca^{2+} stores depends on Ca^{2+} influx through $Ca_v1.2$ channels. The data should indicate that $Ca_v1.2$ channels significantly contribute to Ca^{2+} store refilling after Ca^{2+} store depletion in SMC.

The effective distance between a single L-type $Ca_v1.2$ channel and RyRs within the T-tubular membrane in cardiomyocytes has been estimated to be <100 nm, based on the finding that high concentrations of mobile Ca^{2+} buffers such as EGTA (10 mM) do not disrupt the release of Ca^{2+} sparks by L-type channel openings(Collier et al., 2000), using models of radial diffusion of Ca^{2+} in a concentric shell (Klingauf and Neher, 1997). We will examine the spatial separation of $Ca_v1.2$ channels and RyRs in arterial SMC. To characterize the distance we will determine whether Ca^{2+} sparks disappear in the presence of high concentrations of the membrane-permeable EGTA-AM. These experiments should indicate whether Ca^{2+} ions entering through $Ca_v1.2$ channels at a distance >100 nm from RyRs are required for release of Ca^{2+} sparks in SMC. We next will investigate whether RyRs are able to sense local Ca^{2+} entry by performing experiments in which we will clamp the global $[Ca^{2+}]_i$ at 0.18 nM, 120 nM and 1000 nM, still maintaining high mobile intracellular Ca^{2+} buffer (10 mM EGTA). We expect that STOCs caused by Ca^{2+} sparks are strongly controlled by the global cytosolic $[Ca^{2+}]_i$, with significantly higher frequencies of STOCs at increasing global $[Ca^{2+}]_i$. If STOC frequencies are similar in SMAKO and control cells at identical $[Ca^{2+}]_i$, the results will suggest that local Ca^{2+} influx through $Ca_v1.2$ channels does not activate RyRs to release additional Ca^{2+} sparks.

We expect that the relatively large functional distance between $Ca_v1.2$ channels and RyR (> 100 nm) in arterial SMC enables RyRs to sense global cytosolic $[Ca^{2+}]_i$ to release Ca^{2+} sparks.

Our results will have important implications for the function of Ca^{2+} sparks *in vivo*. Ca^{2+} sparks have been proposed as "negative feedback" regulators(Knot et al., 1998; Nelson et al., 1995) in intact arteries to decrease the global $[Ca^{2+}]_i$ and to limit myogenic tone by membrane hyperpolarization and closure of $Ca_v1.2$ channels through STOCs. In support of a "negative feedback" mechanism, Ca^{2+} spark inhibitors or BK channel blockers depolarize (in a non-additive manner) the cell membrane potential by ~10 mV and increase global cytosolic $[Ca^{2+}]_i$ by ~60 nM(Lohn et al., 2001b). This increase leads to a 30% arterial vasoconstriction(Gollasch et al., 1998; Lohn et al., 2001b) and elevated systemic blood pressure(Brenner et al., 2000; Pluger et al., 2000). However, a "negative feedback" mechanism requires that RyRs specifically tune the release of Ca^{2+} sparks to the needs of an arterial vascular SMC. In the present study, we hope to present strong evidence that sparking RyRs are not activated by local Ca^{2+} influx. Instead, our protocols are intended to show that RyRs sense the global $[Ca^{2+}]_i$ as "common pool" to release Ca^{2+} sparks, which indeed may serve as an "negative feed back" mechanism to oppose vasoconstriction induced by elevations in the global $[Ca^{2+}]_i$.

8. Voraussetzungen für die Durchführung des Vorhabens

All methods are established in the laboratory. All equipment (Nipkow spinning disc confocal microscope, Biorad confocal microscope, two patch clamp setups) necessary for the project is available in the laboratory of Prof. Maik Gollasch (Dept. Nephrology and Intensive Care, CVK, and FVK/MDC-Buch). The PI has developed software to analyze Ca^{2+} sparks and STOCs. He has established the method of tibial and cerebral SMC isolation from SMAKO and wild-type mice.

Mice: All experiments will be conformed to the German animal protection laws. The generation of mice deficient in the smooth muscle $Ca_v1.2$ Ca^{2+} channel (SMAKO, smooth muscle alpha1c-subunit Ca^{2+} channel knockout) has been described recently (Moosmang et al., 2003). Briefly, a conditional *lox*P-flanked allele (L2) of the $Ca_v1.2$ gene (i.e., exons 14 and 15) was generated by homologous recombination in R1 embryonic stem cells. In addition, mice carried a knock-in allele [SM-CreER T2 (ki), (22)], which expresses the tamoxifen-dependent Cre recombinase, CreER T2 , from the endogenous SM22 alpha gene locus, which is selectively expressed in smooth muscle of adult mice. Thus, tamoxifen treatment of mice results in conversion of the *lox*P-flanked $Ca_v1.2$ allele (L2) into a Cav1.2 knockout allele (L1) specifically in SMC. Animals will be kept under standard conditions with water and food ad libitum. At an age of 2 - 3 months, SMAKO mice ($Ca_v1.2$ L1/L2 , SM-CreER T2 (ki) +/.) and corresponding control (CTR) mice ($Ca_v1.2$ +/L2 , SM-CreER T2 (ki) +/.) will be i.p. injected with tamoxifen (2 mg per day) for 5 consecutive days. After 3–4 weeks, mice will be killed by cervical dislocation and the brain and tibial arteries were removed.

Isolation of arterial VSMC: SMC from tibial and basilar arteries will be isolated as described(Gollasch et al., 1998; Pluger et al., 2000). Briefly, the brain and tibial arteries will be removed and quickly transferred to cold (4° C) oxygenated (95 % O_2/ 5 %CO_2) physiological salt solution (PSS) of the following (mM) composition: NaCl, 119; KCl, 4.7; $NaHCO_3$, 25.0; KH_2PO_4, 1.2; $CaCl_2$, 1.8; $MgSO_4$, 1.2; EDTA, 0.026 and glucose, 11.1. The arteries will be cleaned, cut into pieces and placed in a Ca^{2+}-free Hanks solution (in mM): 55 NaCl, 80 Na glutamate, 5.6 KCl, 2 $MgCl_2$, 1 mg/ml bovine serum albumin (BSA, Sigma), 10 glucose and 10 Hepes (pH 7.4 with NaOH) containing 1.0 mg/ml papain (Sigma) and 1 mg/ml DTT for 15 (cerebral arteries) to 45 min (tibial arteries) at 36^{o} C. The segments will then be placed in Hanks solution containing 1 mg/ml collagenase (Sigma, type F and H; ratio 30% and 70%) and 0.1 mM $CaCl_2$ for 6 min (cerebral arteries) to 10 min (tibial arteries) at 36^{o} C. Following several washes in Ca^{2+}-free Hanks solution (containing 1 mg/ml BSA), single cells

will be dispersed from artery segments by gentle trituration. Cells will then be stored in the same solution at 4^{o} C.

Ca^{2+} sparks: VSMC will be seeded onto glass coverslips and incubated with the Ca^{2+} indicator fluo-3-AM (5 µM) and pluronic acid (0.005%; w/v) for 30 min at room temperature in Ca^{2+}-free Hanks solution(Lohn et al., 2000; Lohn et al., 2001a; Pluger et al., 2000). After loading of the cells with fluo-3, the cells will be washed with a HEPES-buffered physiological saline solution (HEPES-PSS) for 30-40 min at room temperature. The HEPES-PSS will have the following composition (in mM): 135 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.4 with NaOH). Single SMC will be imaged using a BioRad (Munich, Germany) laser scanning confocal microscope attached to a Nikon Diaphot microscope(Furstenau et al., 2000; Lohn et al., 2001a). Images will be obtained by illumination with a krypton/argon laser at 488 nm, and recording all emitted light above 500 nm. Ca^{2+} sparks will be measured in HEPES-PSS. Cells will be scanned in the "line scan" mode for 10 s. Ca^{2+} spark analysis will be performed off-line using custom software written in C++ by K. Essin. Ca^{2+} sparks will be defined as local fractional fluorescence increases greater than 1.2. The site of a Ca^{2+} spark will be determined as the center of the spark at the time of its initiation. Ca^{2+} spark width will be determined at 5% maximal amplitude; decay will be measured from peak to half-maximal amplitude. The frequency will be estimated as the number of detected sparks divided by the total scan time. The amplitudes will be expressed as fractional fluorescence increase (F/Fo) or in absolute values relative to the global resting cytosolic $[Ca^{2+}]_r$ using the following equation(Herrera et al., 2001; Jaggar et al., 1998):

$$\Delta[Ca^{2+}]_{spark} = KR/(K/[Ca^{2+}]_r+1-R) - [Ca^{2+}]_r \quad \text{(Eq. 1)},$$

where R is the fractional fluorescence increase (F/Fo), $[Ca^{2+}]_r$ is the free cytosolic Ca^{2+} concentration at Fo, and K is the apparent affinity of fluo-3 for Ca^{2+} (400 nM)(Cheng et al., 1993). $[Ca^{2+}]_r$ will be determined in Fura-2 loaded SMC.

K^+ current recordings: K^+ currents will be measured by the conventional whole-cell or perforated whole-cell patch technique(Gollasch et al., 1996; Lohn et al., 2001a). In perforated patch recordings, whole cell access will be achieved by amphotericin B within 10 min seal formation at room temperature (20-24^{o}C). Amphotericin B (Sigma) will be dissolved in dimethyl sulfoxide (DMSO) and diluted into the pipette solution to 200 µg/ml. Patch pipettes (resistance, 3-5 MΩ) will be filled with a solution containing (in mM): 110 K-aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES, and 0.05 EGTA (pH 7.2). The external solution will contain (in mM): 134 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES (pH 7.4). To clamp the global $[Ca^{2+}]_i$ at different levels, STOCs will be recorded in the conventional whole-cell mode using the following pipette solutions (in mM): 80 K-aspartate, 50 KCl, 10 NaCl, 1 $MgCl_2$, 3 MgATP, 10 Hepes, 10 EGTA and different $[CaCl_2]_i$ (pH 7.2). $[CaCl_2]_i$ will be 0.01, 4 and 8 mM and equaled to 0.18, 120 and 1000 nM free cytosolic $[Ca^{2+}]$. The free cytosolic $[Ca^{2+}]$ will be calculated by the "Cabuf" program written by Prof. G. Droogmans (available at ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip) and based on the stability constants given by Fabiato and Fabiato(Fabiato and Fabiato, 1979). Whole cell currents will be recorded using an EPC 7 amplifier (List, Darmstadt, Germany), digitized at 5 kHz, using a CED 1401 series interface (Cambridge Electronic Design Limited, Cambridge, UK)**,** and CED Patch and Voltage Clamp Software Version 6.08 or an EPC9 amplifier under contol of Pulse software (HEKA electronics, Lambrecht, D). STOC analysis will be performed off-line using custom software written in C++ by K. Essin or ORIGIN 6.1 (Microcal, Northampton, MA, USA).

Recording of global intracellular $[Ca^{2+}]$: Intracellular $[Ca^{2+}]_i$ will be monitored at ~35°C as described(Gollasch et al., 1991). Briefly, isolated tibial myocytes will be loaded with 3 µM Fura-2/AM (www.calbiochem.com) for 30 min in buffer solution (in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, glucose 5.6). $[Ca^{2+}]_i$ will be continuously recorded as fluorescence intensity (at 510 nm) at alternating 350 (F350) and 380 nm (F380) excitation

wavelengths and their respective ratio (F350/F380) by using TILL vision devices (www.till-photonics.de). Stimulation will be performed by local application of caffeine (10 mM) via a syringe device. The resting global cytosolic $[Ca^{2+}]_r$ will be used for estimation of Ca^{2+} spark amplitudes. The absolute amplitudes of Ca^{2+} sparks ($\Delta F/Fo$) relative to the resting cytosolic $[Ca^{2+}]$ will be calculated as $(F/F_O\text{-}1)\cdot[Ca^{2+}]_r$, where F/F_O is local fractional fluorescence increases taken from the experiments with fluo-3 and $[Ca^{2+}]_r$ is the resting global cytosolic Ca^{2+} concentration determined in the experiments with Fura-2.

9. Förderdauer: 1 year

10. Beantragte Mittel und Begründung:

10.1. Investitionsmittel: insgesamt 10.000 Euro für a) Nikon-Präpariermikroskop zum Präparieren der Tibialarterien und Hirnarterien von Wildtyp- und SMAKO-Mäusen, b) Eppendorf-Pipetten für das Applizieren von Lösungen, c) Computer-Auswerteeinheit für STOCs und Ca^{2+}-Sparks. Diese Investitionsmittel sollen aus dem Grundbedarf investiv der Abteilung für Nephrologie und Hypertensiologie gedeckt werden.

10.2. konsumtive Mittel: insgesamt 5.000 Euro für a) Chemikalien (z.B. Kollagenase zum Isolieren von Zellen, Ca^{2+}-Indikatoren (fluo-3, Fura-2), Dihydropyridine, 2-APB, b) Einwegmaterial (Pipettenspitzen, Borosilikat-Kapillaren), c) Speichermedien (CD-ROMs, Wechsellaufwerk, Brenner) zur Datenanalyse, d) Tierstall

10.3. Studentische Forschungsförderung: für eine Gesamtdauer von 10-12 Monaten (dieser Mitarbeiter soll an der Messung von Ca^{2+}-Sparks mitwirken und Versuchslösungen herstellen). Da noch kein(e) Kandidat(in) feststeht, wird der Antrag in 2005 nachgereicht.

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