2. Materials and Methods

2.1. Cell culture

A7r5 smooth muscle cells, derived from embryonic rat thoracic aorta, and human embryonic kidney (HEK293) cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). T-REx-293 cells were obtained from Invitrogen (Karlsruhe, Germany) and RVF-SMC (clonal cell line derived from rat vena cava, cf. Franke *et al*., 1980) from Dr. Franke. Primary cultures of aortic smooth muscle cells derived from newborn Sprague-Dawley or adult Brown-Norway rats were provided by Dr. Reusch (Ives *et al*., 1978; Wilson *et al*., 1993). These will be referred to as neonatal (n)rA-SMC or adult (a)rA-SMC.

All cells were grown in 75 cm^2 plastic tissue culture flasks (Greiner, Frickenhausen, Germany) at 37°C in a humidified atmosphere with 7 % or 5 % CO_2. The following culture media were used for the different cell types:

A7r5 cells and *T-REx-293 cells* (7 % CO_2): Dulbecco's modified Eagle's medium (DMEM, 4.5 g/l glucose) supplemented with 10 % (v/v) fetal bovine serum (Invitrogen), 4 mM L-glutamine (Fluka, Taufkirchen, Germany). For the culture of *T-REx-r6 cells* (T-REx-293 cells stably expressing rTRPC6, see below), 5 µg/ml blasticidin and 250 µg/ml zeocin (both Invitrogen) were added to the culture medium.

HEK293 cells (5 % CO_2): minimum essential medium (MEM, Biochrom, Berlin, Germany) supplemented with 10 % (v/v) fetal bovine serum, 4 mM L-glutamine.

RVF-SMC (7 % CO_2): Dulbecco's modified Eagle's medium (DMEM, 1 g/l glucose) supplemented with 15 % (v/v) fetal bovine serum, 4 mM L-glutamine.

nrA-SMC and arA-SMC (5 % CO_2): minimum essential medium (MEM, Biochrom, 2.2 g/l $NaHCO_3$) supplemented with 10 % (v/v) fetal bovine serum, 4 mM L-glutamine.

100 U/ml penicillin and 100 µg/ml streptomycin (both Biochrom) were added to all culture media.

For Ca^{2+}-imaging and patch clamp recordings, cells were plated onto glass coverslips and used within one to three days after plating. When excised-patch experiments were to be performed, coverslips were coated with poly-L-lysine to ensure cell adherence.

2.2. Molecular biology

2.2.1. Cloning of TRPC5, TRPC6 and chimaeric TRPC6-5

Mouse TRPC5 (cf. Schaefer *et al*., 2000) and chimaeric TRPC6-5, consisting of the N-terminal portion of human TRPC6 (covering the transmembrane core up to the TRP box (EWKFAR)) and the corresponding C-terminal part of murine TRPC5, were provided by Dr. Michael Schaefer.

For the cloning of rat TRPC6, total RNA was prepared from rat brain and A7r5 smooth muscle cells using the TRIzol LS reagent (Invitrogen) according to the standard protocol. cDNA synthesis was performed according to the protocol provided by the manufacturer using 1 µg of total RNA, 200 U of Superscript II reverse transcriptase (Invitrogen) and 5 pM of the primer 5'-CCAGTGAGCAGCAGAGTGACGAGGACT-CGAGCTCAAGCTTTTTTTTTTTTTTTT. For amplification of TRPC6, the following primer set was used:

sense: 5'-CCGGTACCGCCCTTATGAGCCGGGGTAATGAAAACAGAC

antisense: 5'-CCGGATCCCTATCTGCGGCTTTCCTCTTGTTT

TRPC6 was amplified by 30 cycles of PCR using the following conditions: 30 s at 95°C, 15 s at 57°C (annealing temperature), and extension for 210 s at 72°C with Expand-HF polymerase (Roche, Mannheim, Germany), followed by a final extension at 72°C for 10 min. The PCR products were subcloned into the pCR2.1 vector (Invitrogen) and the sequences confirmed by DNA sequencing of both strands (ABI-Prism, Perkin Elmer). The obtained sequence corresponds to rat TRPC6B published by Zhang & Saffen (2001, GenBank™ accession number: AB051213) with the exception of two amino acid exchanges: M757I and S767F.

2.2.2. Transfections

2.2.2.1. Stable expression of TRPC6

For the generation of a stably-transfected, inducible cell line, the Kpn I/ BamH I fragment of the cloned cDNA was ligated into the tetracycline-inducible eukaryotic expression vector pcDNA4/TO (Invitrogen) and transfected into T-REx-293 cells using the FuGENE 6 transfection reagent (Roche). Clonal selection was performed according

to the manufacturer's protocol with Zeocin (250 µg/ml). Cells were induced with tetracycline (1 µg/ml, Roche) for one to two days and 36 clones were functionally screened for rTRPC6 expression with electrophysiological methods (AlF_4^- infusion, whole-cell recordings). Two positive clones were selected and used for further analysis.

2.2.2.2. Transient transfection

For transient transfection, cells were seeded in 35-mm culture dishes. The following day, a mixture of 2 µg/dish of pcDNA3 vector containing either the cDNA for mTRPC5 or that for the chimeric TRPC6-5, 100 ng/dish of the rat histamine H_1 receptor (in pcDNA3), and 50 - 100 ng/dish of pEGFP-C1 (CLONTECH, Palo Alto, CA), a construct containing the green fluorescent (GFP) marker gene, were transfected into HEK293 cells using the FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol. For some experiments, T-REx-r6 cells were transiently co-transfected with the rat histamine H_1 receptor using 100 ng/dish of the rat histamine H_1 receptor (in pcDNA3) mixed with 50 - 100 ng/dish of pEGFP-C1, using the above protocol. After 18 - 24 h, the transfected cells were trypsinized and seeded onto glass coverslips. For electrophysiological or fluorometric studies, only cells displaying green fluorescence when excited with light of 490 nm were used. All experiments were performed 2 - 3 days after transient transfection and, in the case of T-REx-r6 cells, 1 - 2 days after induction with tetracycline.

2.2.3. Northern hybridization

2.2.3.1. Basic principles

Northern hybridization is a commonly used laboratory technique designed to obtain information on cell or tissue distribution of specific m(essenger)RNA molecules. It also allows the size and relative amounts of the respective molecules to be determined. The procedure has been termed northern hybridization or northern blotting to contrast it with the corresponding Southern technique for DNA analysis that was developed by E. M. Southern.

For northern blotting, total RNA or purified mRNA can be used. The use of total RNA is less satisfactory because nonspecific binding of the probe used for detection to the highly abundant r(ibosomal)RNAs may lead to a substantial background signal. Northern hybridization involves, as first step, RNA separation by gel electrophoresis. Because single-stranded RNA can form secondary structures, samples must be electrophoresed under denaturating conditions, by adding e.g. formaldehyde to the agarose gel. Under these conditions, the separation of RNA only depends on the total length of the molecules. Subsequent transfer ('blotting') of the separated RNA from the agarose gel to an adsorbent membrane (nitrocellulose filter or nylon membrane) is achieved by capillary action (cf. Fig. 4). When transferred to the membrane, the RNA bands stay in the same relative positions as on the gel. After immobilizing of the RNA on the membrane, the membrane can be hybridized, i.e. bathed in a solution containing a labeled probe that is complementary to the sequence of interest. Radioactively-labeled cDNA probes are routinely used. After removal of the unbound or nonspecifically bound probe, autoradiographic analysis allows detection of labeled bands at positions determined by the size of the RNAs containing the complementary sequence. Usually, housekeeping genes, that are expressed to a similar extent in all cells, are used as a control for RNA integrity and to obtain estimates for the relative amounts of the specific mRNAs.

Probes for northern hybridization are ideally 100 to 1000 bp in length. When designing specific probes that discriminate between mRNA of related genes, it is useful to select either segments of the coding sequence that display low sequence similarity among family members, or to include untranslated regions because these sequences are less conserved among related genes.

Northern blotting is widely used for studying the regulation of transcription and gene expression. However, like PCR expression studies, it can also be applied instead of protein expression studies (Western blotting), when specific antibodies for the proteins under investigation are not available. It is less sensitive than PCR techniques, but does not involve any amplification steps which may lead to errors. It has to be kept in mind than that the amounts of the mRNAs obtained in northern blot experiments do not necessarily reflect the amounts of the respective proteins.

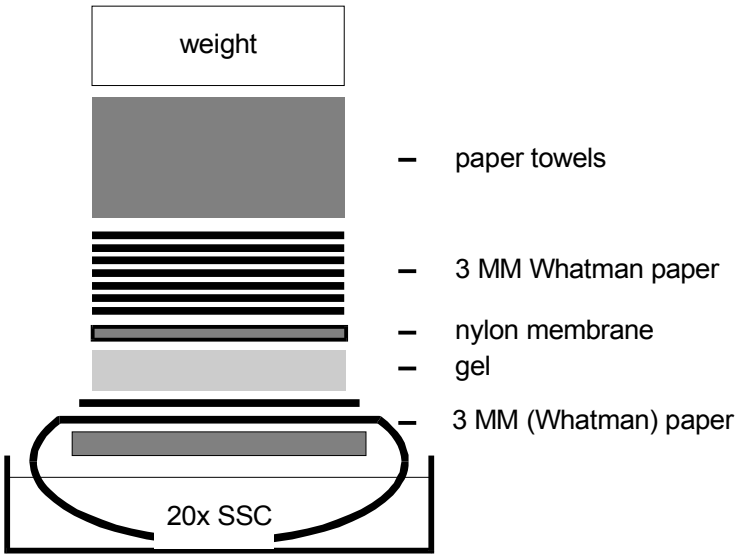

Fig. 4: **RNA transfer set up.** The RNA is carried along with the fluid flow, which builds up due to capillary action, and becomes trapped by the nylon membrane in the same relative position that it occupied in the gel.

A major difficulty of northern hybridization studies is the risk of RNA degradation by contamination with ribonucleases (RNases). These are very stable enzymes that do not require cofactors and, thus, are difficult to inactivate. Hence, precautions have to be taken to avoid contamination.

2.2.3.2. RNA isolation

For RNA isolation, A7r5 cells or RVF-SMC cells were harvested by trypsinization and subsequent centrifugation, and were immediately frozen in liquid nitrogen. Brain and vomeronasal organ (VNO) were freshly prepared from female Wistar rats (obtained from Dr. Klug), immediately frozen in liquid nitrogen and subsequently pulverized at -70°C using a mortar and pistil. The pellet of harvested cells or, alternatively, the pulverized tissue was resuspended in TRIzol LS (Invitrogen) and homogenized. Total RNA was purified from either probe according to the manufacturer's protocol. After redissolving the RNA in RNAase-free water (DEPC-treated, see below), the RNA yield was determined in a photometer using the absorption at 260 and 280 nm. To ensure that the purified RNA had not been degraded during the isolation procedure, samples of the purified RNA were separated by gel electrophoresis using standard 1 % agarose gels

containing ethidium bromide. The occurrence of two distinct bands corresponding to the 28S and 18S ribosomal RNAs was used as a marker for good quality mRNA.

Detection of TRPC1 mRNA was only possible after purification of mRNA from the total RNA. Since messenger RNAs generally contain a poly(A) tail, while structural RNAs (rRNA or tRNA) do not, oligo(dT) columns (Oligotex Direct mRNA kit, Quiagen, Hilden, Germany) were used for enrichment of mRNA.

2.2.3.3. Amplification of isotype-specific probes and probe-labeling

1 - 5 µg of total RNA from rat brain were reverse transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen) and 5 pM of primer 5'-CCAGTGAGCAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTT TTTTT. The obtained cDNAs were used as templates for subsequent PCR amplification.

Since the nucleotide sequences of the 3'-untranslated regions (3'-UTR) were not available for all rat TRPC isoforms, 3'-RACE (**r**apid **a**mplification of **c**DNA **e**nds) was performed for rTRPC2, rTRPC4, rTRPC5 and rTRPC7. The following nested oligonucleotides that match the reverse transcription primer were employed as antisense primers in the 3'-RACE: CCAGTGAGCAGAGTGACG (outer primer) and GAGGAC-TCGAGCTCAAGC (inner primer). Nested amplification of the 3' ends of the cDNA was performed in two rounds of 25 cycles under the following conditions: annealing temperature: 55°C (15 s), elongation time 150 s (72°C), Expand HF DNA polymerase (Roche). The following oligonucleotides were used as sequence-specific sense primers:

target	**outer primer**	**inner primer**
rTRPC2	5'-CAGACTGACAGAGCTGACCAAGAC	5'-TGACACCCCCGCAGAGC
rTRPC4	5'-GCTGAGCAAAACGCAAACCA	5'-CGGCAGGAGCACTTGAGAGA
rTRPC5	5'-GGCAGAGGCCTGTTCGCAAA	5'-GTGCTGCTCGGAGCAGTGAA
rTRPC7	5'-TGAAAACCTGACAGCCAATAGCAC	5'-TTCAGCAAGCCCACCAGATACC

The amplificates were subcloned into the pCR2.1 vector (Invitrogen) and the sequence determined by automated cDNA sequencing of both strands (ABI prism).

The obtained sequences and the available 3'-UTR sequences of rTRPC1, rTRPC3 and rTRPC6 were used to amplify fragments covering parts of the C-terminal coding sequence and the adjacent untranslated region. The following primer sets were used for the amplification of specific northern probes:

rTRPC1: 5'-GGGAATTCGCCCCGGCTACACAGAGAAACC (sense)/ 5'-GGGAATTCGGGAACACTTAAACCTGGGACATT (antisense), **rTRPC2**: 5'-GGGAATTCA-GGGCCAGCTGGGTCTCGTGGGGAATTCGCCCCGGCTACACAGAGAAACC (sense)/ 5'-GGGAATTCATGCCCCGGTAGGTGTTGATTC (antisense), **rTRPC3**: 5'-GGGAATTCGAACTGGGCATGGGTAACTCAAAGGGGAATTCGCCCCGGCTACACAGAGAAACC (sense)/ 5'-GGGAATTCCGTCATGGCTGGTCGTAAAACA (antisense), **rTRPC4**: 5'-GCTGAGCAAAACGCAAACCAGGGAATTCGCCCCGGCTACACAGAGAAACC (sense)/ 5'-AATTATTACGTCAAGATGGATGGAAGT (antisense), **rTRPC5**: 5'-GGCATCGCACAGCAGCACTCTATGGG AATTCGCCCC-GGCTACACAGAGAAACC (sense)/ 5'-CAGCATGGGCAGCGTGTAAGC (antisense), **rTRPC6**: 5'-CGGTGGTCATCAACTACAATCAGGGAATTCGCCCCG-GCTACACAGAGAAACC (sense)/ 5'-TCCAAATGATCCAAGTTACCAGTT (antisense), **rTRPC7**: 5'-TCCCTTTAACCTGGTGCCGAGTCGGGAATTCGCCCC-GGCTACACAGAGAAACC (sense)/ 5'-ATGTGCGTATGTTGGGGAGGAA (antisense)

Cycling conditions were: 2 min at 95°C, followed by 10 cycles of 30 s at 95°C/ 15 s at 55 - 65°C/ 60 - 70 s at 72°C, followed by 20 cycles of 30 s at 95°C/ 15 s at 55 - 65°C/ 60 - 70 s + 5 s/cycle at 72°C, and a final extension at 72°C for 7 min. The PCR products were subcloned into pCR2.1/TOPO or pcDNA3.1/TOPO vectors (Invitrogen) and the sequences were confirmed by DNA sequencing of both strands (ABI Prism). Depending on the vector used and on the presence of internal restriction sites, EcoRI, BSTX1 or NotI-fragments (New England Biolabs GmbH; Frankfurt/Main, Germany) were radiolabeled by random priming (Prime-It Rmt random primer labeling Kit, Stratagene, Amsterdam, The Netherlands) with [α-^{32}P]dCTP (NEN Life Science Products,

Zaventem, Belgium) and purified by gel filtration (ProbeQuant, Amersham Pharmacia Biotech Europe, Freiburg, Germany).

2.2.3.4. RNA gel electrophoresis and blotting

40 µg of total RNA (or purified mRNA in the case of TRPC1) from the desired cells or tissues (48 µg of VNO total RNA) were diluted with RNase-free water to give a final volume of 10 µl. 50 µl of electrophoresis sample buffer (see below) were added, the mixture was incubated for 10 min at 65°C and transferred onto ice until use. 5 µg molecular weight standards (RNA Millenium™ size markers, Ambion, Huntingdon, UK) were prepared accordingly. RNA samples were separated by gel electrophoresis on a formaldehyde/1.2 % agarose gel (see below) using RNase-free 1x MOPS running buffer (see below). To remove possible contamination by RNases, the gel apparatus was treated with RNaseZAP (Ambion) and rinsed twice with RNase-free water before use. After successful gel electrophoresis (~ 50 V for 2 - 3 h), the gel was incubated for 20 min in 0.05 M NaOH in 1x standard salt saline (SSC, see below) followed by two soaking periods of 10 min in 10x SSC. The mild NaOH treatment increases transfer efficiency without greatly degrading the RNA. The RNA was transferred from the gel to a nylon membrane (Hybond-XL, Amersham Pharmacia Biotech Europe or Tropilon-Plus, Applied Biosystems, Weiterstadt, Germany) using the transfer set up shown in Figure 4. The nylon membranes were soaked in 10x SSC before use. Transfer was done overnight. The next day the membrane with the bound RNA was retrieved from the transfer set up and incubated in 10x SSC for 5 min. The RNA was fixed on the membrane by cross-linking in an UV transilluminator (Stratagene). After cross-linking, blot membranes were stained using methylene blue (see below) as a control for the integrity and the amount of the transferred RNA. Furthermore, methylene-blue staining served to obtain the position of the RNA size standards on the blot. The membrane was dried by baking for 2 h at 80°C and kept at room temperature until use.

2.2.3.5. Hybridization and analysis

Four to five hours of prehybridization in UltraHyb hybridization solution (Ambion) were followed by overnight hybridization at 42 - 50°C using the same solution

supplemented with $1x10^6$ cpm/ml of labeled probe. Blots were washed using high-stringency washing protocols (twice with 2x SSC/0.1% SDS at 50°C for 10 min, 60 min in 0.2x SSC/0.1% SDS at 50°C, twice with 0.1x SSC/0.1% SDS at 55 - 60°C for 60 min). Autoradiographs were obtained using a phosphoimaging system (BAS-reader, Fuji) and exposure times of at least 24 h. After autoradiographs had been obtained, blots were stripped (stripping solution and conditions, see below) and rehybridized with a 888-bp cDNA fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.2.3.6. Materials

Chemicals

Chemicals were guaranteed to be RNase-free by the supplier and were only used for RNA experiments. Diethyl pyrocarbonate (DEPC), NaCl, sodium acetate, 3-[N-morpholino]propanesulphonic acid (MOPS), Tris-(hydroxymethyl)aminomethane (TRIZMA Base, Tris), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulphate (SDS), formaldehyde (37 %), and glycerol were purchased from Sigma (Taufkirchen, Germany). Sodium citrate and deionized formamide were obtained from Calbiochem (Bad Soden, Germany) or Merck (Darmstadt, Germany), respectively. Bromophenol blue was from SERVA Electrophoresis GmbH (Heidelberg, Germany) and Saekem LE agarose from BioWhittaker Molecular Applications (Rockland, USA).

Solutions and gels

RNase–free water: 0.1 % DEPC was added to deionized water, incubated for 2 h at room temperature and then autoclaved. 10x MOPS: 0.2 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA (pH 7.0 with NaOH). 20x SSC: 3 M NaCl, 0.3 M sodium citrate (pH 7.0 with HCl) Electrophoresis sample buffer: 0.75 ml deionized formamide, 0.15 ml 10x MOPS, 0.24 ml formaldehyde, 0.1 ml deionoized RNase-free H_2O, 0.1 ml glycerol, 0.08 ml 1 % (w/v) bromophenol blue

Methylene blue solution: 0.04 % methylene blue, 0.5 M sodium acetate. Stripping conditions: Two different protocols were used. 3 x 60 min wash at 85°C in a solution containing 0.1x SSC, 1 % SDS, 40 mM TrisCl or 2 x boiling 0.1 % SDS in H_20 with 15

to 30 min cooling periods and subsequent wash in 2x SSC without SDS
Formaldehyde/agarose gel: 1.2 g agarose, 100 ml 1x MOPS, 6 ml formaldehyde.
Precautions taken to avoid RNase contamination
Labware used in the preparation of RNA was treated as follows to remove residual RNase activity: glassware was baked at 250°C for up to 8 h. Disposable plasticware was used straight out of the package. Plasticware that was repeatedly needed was treated with RNaseZAP and rinsed with RNase-free water before use.

2.3. Patch clamp recordings

2.3.1. Basic principles

Electrophysiological techniques are used to study ion channel function by recording either the currents that flow through open ion channels or the changes in membrane potential that result from these ion fluxes. In the present work, the patch clamp technique was applied to examine receptor-operated cation currents through endogenous or recombinant channel proteins. The patch clamp technique was developed by Erwin Neher and Bert Sakmann (Neher & Sakmann, 1976, Hamill *et al*., 1981) and is widely used to record whole-cell currents from small cells as well as to measure the activity of single ion channels (see Sakmann & Neher, 1983; Ogden & Stanfield, 1994; Numberger & Draguhn, 1996). In patch clamp recordings, a patch of membrane is electrically isolated from the surrounding solution by pressing an electrolyte-filled glass micropipette (tip diameter ~ 1 µm) against the surface of the cell and subsequently applying suction. A silver/silver chloride electrode couples the electrolyte in the micropipette to a special electrical circuit (Fig. 5*A*). Under ideal conditions, a very tight seal between the cell membrane and the glass wall of the pipette is formed (seal resistances above 1 GΩ, *gigaseal*). This allows currents flowing through single ion channels in the patch of membrane spanning the pipette tip to be resolved. The configuration with the pipette sealed to the membrane of an intact cell is called *cell-attached* configuration (Fig. 5*B*). It is used to study single-channel currents under physiological conditions. Disadvantages arise from the fact that the cell is still intact. Thus, the composition of the intracellular solution and the patch potential are unknown.

Because of the high mechanical stability of the seal, three other recording configurations can be obtained (Fig. 5*B*).

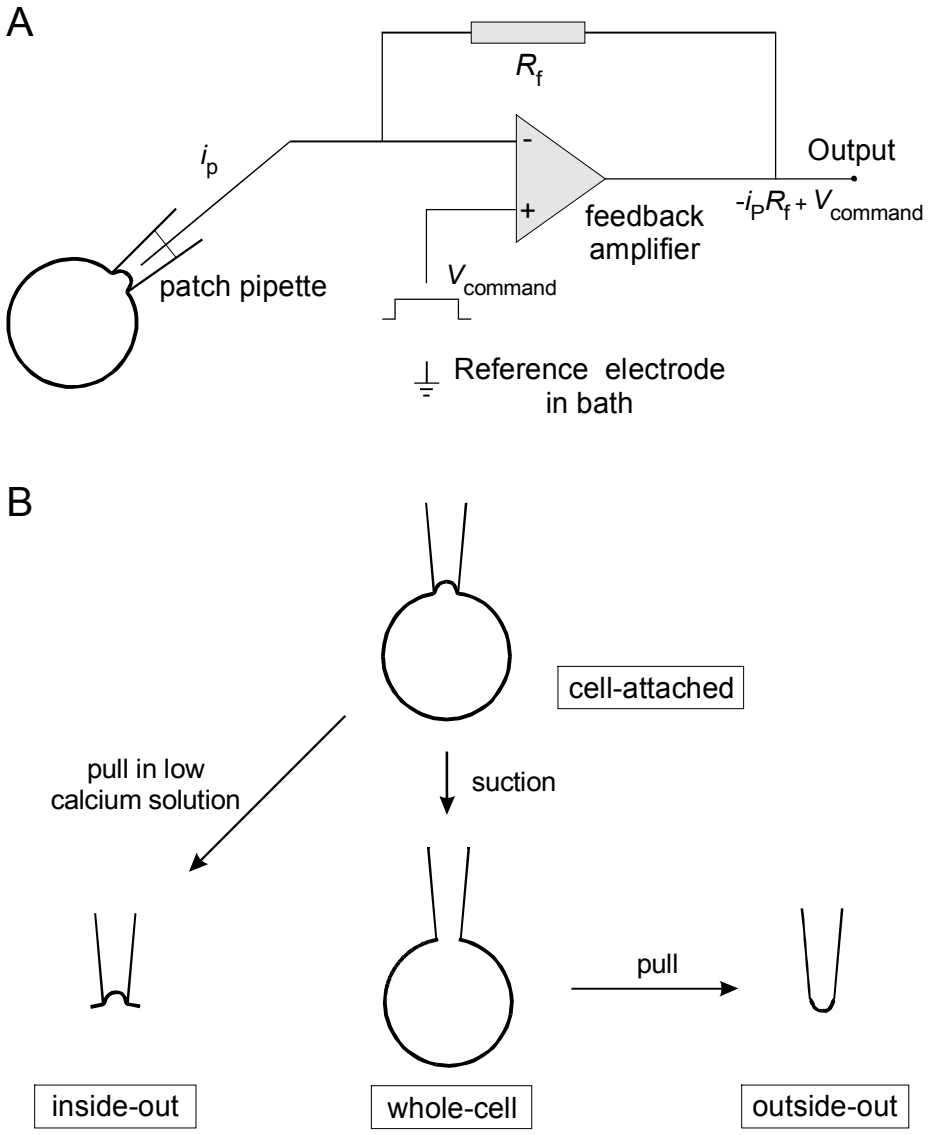

Fig. 5: *A*: **Patch clamp circuit.** Main features of the headstage amplifier used in patch clamp experiments. The patch pipette is connected to the inverting input (-) of a feedback amplifier. The non-inverting input (+) of the amplifier is connected to a variable voltage source and can be set at a desired value (command potential, $V_{command}$). In the case of a voltage difference between the two inputs of the amplifier, current is delivered from its output so that the patch is clamped at the command potential. The current through the patch (i_p) is measured as the voltage drop (V_p) across the feedback resistor (R_f). *B*: **Patch clamp configurations.**

Inside-out: The membrane patch is pulled off the cell into the bath solution with the intracellular surface facing the bath solution. Thus the properties of single-channel

currents can be recorded under defined ionic conditions and with full control of the potential across the patch. The solution bathing the intracellular surface of the isolated membrane patch can be easily exchanged. *Inside-out* patches are, therefore, used to test the effects of putative cytosolic regulators of channel activity. Since cytosolic components may be lost in the *inside-out* configuration, the activity of ion channels may show run-down.

Whole-Cell: The membrane patch under the pipette tip is destroyed by applying suction to the pipette. Thus, low-resistance electrical access to the cell interior is gained. Single-channel currents cannot usually be resolved in the whole-cell configuration. Instead, the summed activity of all open ion channels in the entire cell membrane (i.e. the whole-cell current) is measured. In the whole-cell configuration the intracellular solution can be manipulated, but soluble cytosolic constituents may be lost from the cell. Current fluctuations arising from the summation of the many independent, randomly occurring openings and closings of the underlying single channels can be used to derive mathematically some information on the single-channel properties (*fluctuation analysis*).

Outside-out: The pipette is withdrawn in the *whole-cell* configuration resulting in a patch of membrane whose extracellular surface is exposed to the bath solution. This configuration is especially useful to study the effect of extracellular modulators of channel activity. For advantages and disadvantages see *inside-out* configuration.

2.3.2. Recording conditions for patch-clamp experiments

For electrophysiological recordings, cells were placed in a recording chamber (chamber volume ~ 0.5 ml) and continuously superfused with solution by gravity feed at a rate of 3 ml/min. All experiments were performed at room temperature (20 - 25°C). Only single cells not coupled to any neighbouring cells were used in the experiments. Whole-cell and single-channel recordings were performed using an EPC-7 amplifier and Pulse software (HEKA, Lambrecht, Germany). Patch pipettes were made from borosilicate glass and, when filled with the standard intracellular solutions, had resistances of 3 - 6 MΩ for whole-cell recordings or 6 - 9 MΩ for single-channel recordings.

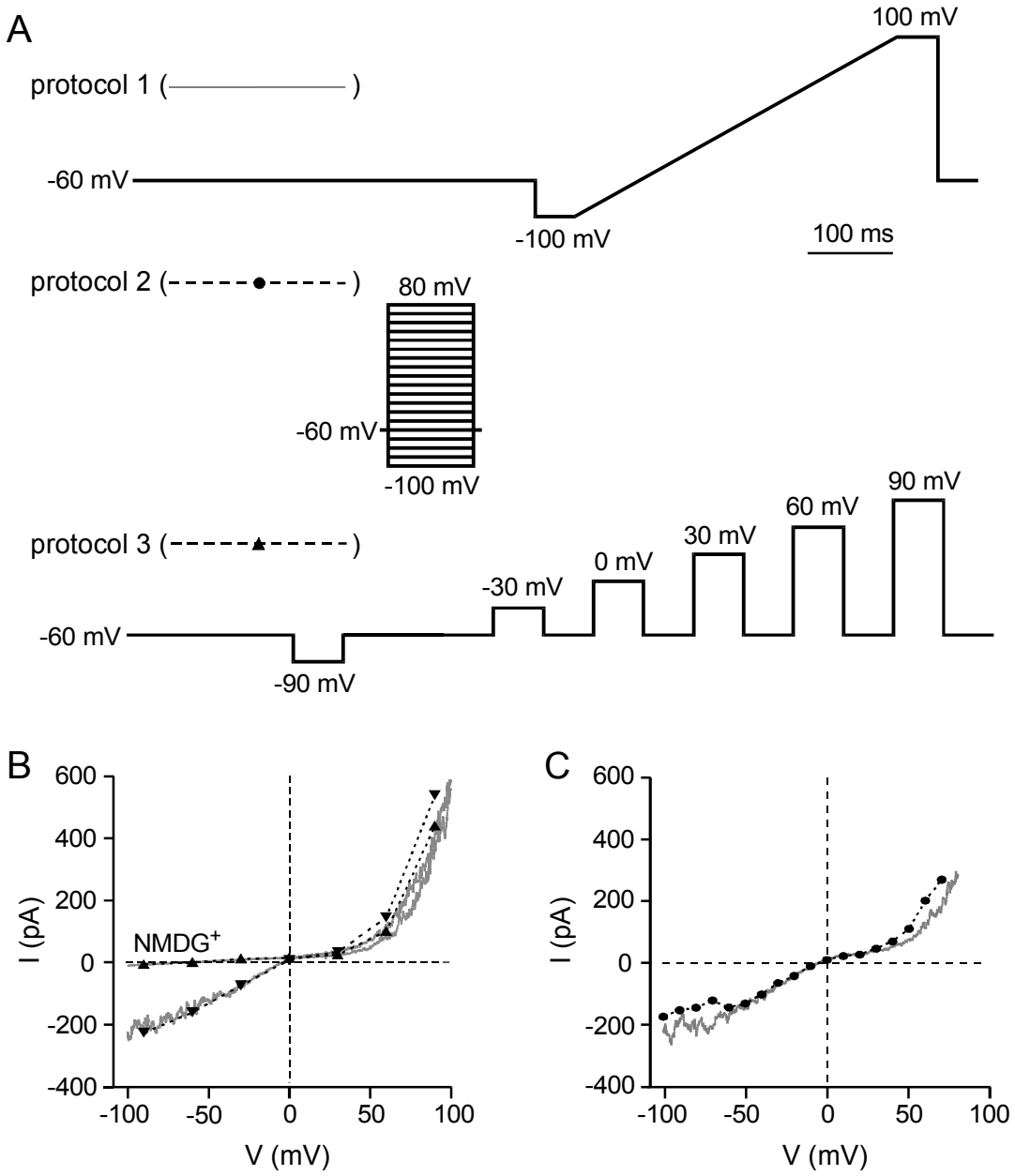

Fig. 6: **Comparison of the *I-V* relationships of vasopressin-induced whole-cell currents in A7r5 cells obtained by three different protocols.** *A*: Voltage protocols used to determine the current-voltage (*I-V*) relationship of whole-cell currents in A7r5 smooth muscle cells. *Protocol 1*: From a holding potential of -60 mV the voltage is stepped to -100 mV and than changed continuously to +100 mV at a rate of 0.5 V/s. *Protocol 2*: From the holding potential of -60 mV the voltage is stepped for 100 ms to different levels between –100 and +80 mV and back to -60 mV. *Protocol 3*: Similar to *protocol 2,* but with shorter intervals and pulses. *B,C*: Comparison of the *I-V* relationships of AVP-induced cation currents obtained with the protocols shown in *A*.

For standard whole-cell recordings, cells were held at a potential of -60 mV, and current-voltage (*I-V*) relations were routinely obtained every 5 s from voltage ramps from -100 to +100 mV with a duration of 400 ms. Ramp data were filtered at 1 kHz and

acquired at a frequency of 4 kHz. To exclude artefacts introduced into the *I-V* relations of the vasopressin-induced currents in A7r5 cells by the ramp protocol (where voltage is continuously changed), additional voltage protocols (Fig. 6*A*) were tested. The resulting *I-V* relations were overlaid for reasons of comparison (Fig. 6*B*) and found to be similar irrespective of the protocol used. The holding current was continuously acquired at 30 Hz. Series resistance (R_s = 6 - 15 MΩ) was not compensated. For fluctuation analysis of A7r5 whole-cell currents, the holding current was filtered at 5 kHz and sampled at 10 kHz. Current variance (σ^2) was plotted against the mean current amplitude (I), and the single-channel current (i), as well as the total number of channels in the patch (N), were estimated by fitting the equation $\sigma^2 = iI\text{-}I^2/N$ to the plots. The single-channel chord conductance (γ) at -60 mV was calculated from the single-channel current using Ohm's law.

For single-channel recordings, the standard excised outside-out patch configuration was used. Single-channel data were filtered at 10 kHz and initially recorded onto digital audio tape (DAT) using a digital tape recorder (Biologic, Claix, France). Single-channel recordings were generally carried out at a holding potential of -60 mV. To evaluate the unitary single-channel current-voltage (*I-V*) relationship of recombinant TRPC5, TRPC6 and chimaeric TRPC6-5, the pipette potential was stepped from -60 mV to potentials between –100 and +80 mV. For off-line analysis, the single-channel data were filtered at 1 kHz and subsequently digitized at 15 kHz.

2.3.3. Data analysis

Whole-cell currents

To quantify current stimulation and current inhibition, currents before and after application of the modulating substance were interpolated and the values obtained in the presence of the modulator were normalized to the interpolated values. This method was used to avoid errors arising from the fact that the endogenous agonist-induced cation currents in A7r5 cells as well as recombinant TRPC5 and TRPC6 currents decay over time. All current amplitudes were calculated as the difference between resting and agonist-induced current levels. For calculation of mean current densities, current

amplitudes were normalized to the cell capacitance. When possible I-V relations were leak-corrected by substracting the I-V of resting currents from that of the activated cation currents. The concentration-response curve obtained for lanthanum-inhibition of TRPC6 currents was fitted using to the Hill equation $y = 1 - 1/(1 + (IC_{50}/x)^n)$ to determine the IC_{50} value. n is the Hill coefficient.

Single-channel currents

The analysis was performed using the pClamp6 software (Axon Instruments, Foster City, CA). Mean single-channel amplitudes were obtained by scanning the data traces manually and using the measuring options provided by pClamp6. Only amplitudes of events with open durations of more than 2 ms were used. In the case of TRPC5, channel activity was expressed as NP_o, the product of the minimum number (N) of channels in the patch (obtained from the observed number of open levels) and the open probability (P_o). NP_o values were calculated for consecutive 2-s periods. Openings with durations shorter than 0.5 ms were excluded from the analysis. Because of extensive overlap of individual unitary current responses after application of lanthanides, an algorithm established by Fenwick et al. (1982) was used to obtain a reliable estimate of mean open times in the absence and presence of lanthanides. The general applicability of the algorithm has been confirmed (Ramanan et al., 1992). The estimate of mean channel open time (t_o) was calculated according to $t_o = (\Sigma\ jt_j)/N$ where N is the number of all channel openings (transitions between a given level j and a subsequent level j + 1), t_j designates the dwell time of a given level j and the sum extends over all levels encountered in the recording. Values for t_j were extracted from idealized traces generated with pClamp6 and fed into Microsoft Excel to do the final calculation. For TRPC6, calculations of NP_o proved difficult because most openings were extremely short often below the minimum resultion of 0.5 ms (about twice the rise time of a 1 kHz filter). To circumvent this problem, the frequency of channel openings was determined. The frequency values were calculated for consecutive 5-s periods.

2.3.4. Solutions

The standard extracellular solution contained 140 mM NaCl, 5 mM CsCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). The standard intracellular solution contained 110 mM cesium methanesulphonate, 25 mM CsCl, 2 mM $MgCl_2$, 3.62 mM $CaCl_2$, 10 mM EGTA, and 30 mM HEPES (pH 7.2 with CsOH) with a calculated [Ca^{2+}] of 100 nM. In some experiments, a highly Ca^{2+} buffered pipette solution (30 mM BAPTA) with a calculated [Ca^{2+}] of 100 nM was used. It contained 50 mM cesium methanesulphonate, 25 mM CsCl, 2 mM $MgCl_2$, 9.73 mM $CaCl_2$, 30 mM BAPTA and 30 mM HEPES (pH 7.2 with CsOH). The osmolarity of all solutions was between 290 and 310 mosmol/l. For aluminium fluoride (AlF_4^-) infusion, 1 µl NaF (0.5 M) was mixed with 0.5 µl $AlCl_3$ (3 mM) and diluted with 50 µl of the pipette solution.

2.4. Fluorometric $[Ca^{2+}]_i$ measurements

2.4.1. Basic principles

Fluorescent ion indicator dyes selectively bind certain ion species and, upon binding, exhibit altered fluorescent properties. When loaded into living cells, these fluorescent probes can be used to monitor dynamic changes in the intracellular concentration of the respective ion.

The pivotal importance of Ca^{2+} in cellular signal transduction is reflected by the fact that more studies have been done with fluorescent indicators for Ca^{2+} than for any other ion. Since its development by Roger Tsien and collaborators in 1985 (Grynkiewicz *et al*., 1985), fura-2 has become the most widely used fluorescent probe for measuring $[Ca^{2+}]_i$ changes in single cells. It should be noted, however, that because of its relatively high affinity to Ca^{2+} (in *vitro* K_d = 224 nM, Grynkiewicz *et al*., 1985), fura-2 based Ca^{2+} imaging is only suitable for measuring $[Ca^{2+}]_i$ changes between 20 nM and 2 µM (see Takahashi *et al*., 1999). Ca^{2+} binding to fura-2 leads to a shift of the fura-2 excitation spectrum towards shorter wavelengths. By contrast, the peak emission of fura-2 does not shift significantly with Ca^{2+} binding. As a consequence, the ratio of the fluorescence intensities obtained successively at two excitation wavelengths provides a good measure

of $[Ca^{2+}]_i$. It is independent of dye concentration, cell thickness, excitation light intensity, dye leakage, photobleaching, as well as camera sensitivity (Grynkiewicz *et al*., 1985), because these should affect the measurements at both excitation wavelengths to the same extent. Typically, fura-2 is excited at 340 nm and 380 nm and fluorescence emission is recorded at 510 nm. In this setting, the largest dynamic range is achieved. The fluorescence emission during excitation at 380 nm (F_{380}) decreases upon binding of Ca^{2+} whereas the fluorescence emitted during excitation at 340 nm (F_{340}) increases. Thus, the ratio F_{340}/F_{380} obtained by dividing the fluorescence emitted during excitation at 340 nm by that emitted during excitation at 380 nm changes in the same direction as $[Ca^{2+}]_i$.

Quantitative estimates of $[Ca^{2+}]_i$ can be made from the ratio F_{340}/F_{F380} using the dissociation constant of fura-2, that can be derived from calibration curves (see Grynkiewicz *et al*., 1985). For estimates to be accurate, *in situ* Ca^{2+} calibration is necessary because calibration constants change as a function of various parameters such as e.g. viscosity, ionic strength or temperature of the probe environment (Malgaroli *et al*., 1987; Williams & Fay, 1990; Petr & Wurster, 1997). Accurate assessment of these calibration parameters *in situ* is time-consuming and above all prone to many difficulties and errors. Since absolute $[Ca^{2+}]_i$ changes were of no interest, calibration was omitted in the present study. Because the relationship between $[Ca^{2+}]_i$ and F_{340}/F_{F380} is essentially linear for ratio values between 0 and 2.5, estimates of relative changes in $[Ca^{2+}]_i$ were, nonetheless, possible.

Divalent cations that are not normally found in cells, such as e.g. Mn^{2+}, can permeate a variety of Ca^{2+}-permeable channels in the plasma membrane. These cations frequently interact with fura-2 (Takahashi *et al*., 1999). In the case of Mn^{2+}, the affinity of fura-2 for this cation is 42-fold higher than for Ca^{2+} (Grynkiewicz *et al*., 1985). In contrast to the effects of Ca^{2+} on fura-2, binding of Mn^{2+} causes quenching of fura-2 fluorescence at all wavelengths. Fura-2 fluorescence at 358 nm excitation is independent of $[Ca^{2+}]$ (isosbestic wavelength). Hence, in the presence of Mn^{2+}, fluorescence emission at 358 nm excitation can be used to monitor Mn^{2+} entry. These so-called *manganese quench experiments* allow divalent cation entry pathways to be directly resolved provided that the entry pathway under investigation is permeable to Mn^{2+}.

2.4.2. Recording conditions for Ca^{2+} imaging experiments

A7r5 cells or HEK293 cells were loaded in standard extracellular solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4 with NaOH) supplemented with 4 µM fura-2 AM and 0.005 % Pluronic F-127 at room temperature for 60 – 120 min or 30 – 60 min, respectively. Cells were then rinsed with standard extracellular solution and allowed to de-esterify for at least 30 min at room temperature. Measurements of $[Ca^{2+}]_i$ were performed using an inverted microscope (Axiovert 10, Zeiss, Göttingen, Germany) equipped with an imaging system (T.I.L.L. Photonics, Gräfeling, Germany). The illumination was generated by a xenon arc lamp and a monochromator. The excitation wavelength was alternated between 340 and 380 nm, and the fluorescence emission of selected areas within the A7r5 cell layer was long-pass filtered at 520 nm and recorded every 5 s with a CCD camera. After correction for background fluorescence, the fluorescence ratio F_{340}/F_{380} was calculated. For *manganese quench experiments*, fura-2 fluorescence was also excited at the isosbestic wavelength (358 nm). Fluorescence emission at 358-nm excitation was normalized after correction for background fluorescence to the mean fluorescence values recorded at the beginning of the experiment before addition of Mn^{2+} to the bath solution. Monochromator settings and data acquisition were controlled by Fucal 5.12 C software (T.I.L.L. Photonics). All experiments were performed at room temperature. Coverslips were mounted in a recording chamber on the microscope stage (chamber volume ~ 0.5 ml) and continuously superfused at a rate of ~ 5 ml/min using gravity feed. For Ca^{2+}-free solutions, Ca^{2+} was replaced by 1 mM EGTA. All experiments were performed at room temperature.

2.5. Chemicals

[^{8}Arg]-vasopressin, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), Bay K8644, cobalt chloride, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), flufenamate, gadolinium chloride, GTPγS, heparin, N-2-hydroxyethylpiperazine-N'-(2-ethanesulphonic acid) (HEPES), 5-hydroxytryptamine (serotonin), $InsP_3$, lanthanum chloride, manganese chloride, nickel

chloride, niflumic acid, nimodipine, 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 1-oleoyl-2-acetyl-*sn*-glycerol (OAG), phorbol 12-myristate 13-acetate (PMA), and SKF 96365 were obtained from Sigma. Fura-2 and Pluronic F-127 were purchased from Molecular Probes (Eugene, OR, USA). Thapsigargin was from Alomone Labs (Jerusalem, Israel) and platelet-derived growth factor (rhPDGF-BB) from Calbiochem. Different batches of arachidonic acid from Sigma and from Cayman Chemical Company (Ann Arbor, Michigan, USA) were used. Stock solutions were made in water or DMSO and diluted to final concentrations with respective solutions.

2.6. Statistics

Averaged data are given as means ± S.E.M. The statistical significance of differences between mean values was assessed using Student's *t* test. Differences were regarded as statistically significant for $P < 0.05$.