Mammalian members of the classical transient receptor potential channel (TRPC) subfamily (TRPC1–7) are Ca\(^{2+}\)-permeable cation channels involved in receptor-mediated increases in intracellular Ca\(^{2+}\). Unlike most other TRP-related channels, which are inhibited by La\(^{3+}\) and Gd\(^{3+}\), currents through TRPC4 and TRPC5 are potentiated by La\(^{3+}\). Because these differential effects of lanthanides on TRPC subtypes may be useful for clarifying the role of different TRPCs in native tissues, we characterized the potentiating effect in detail and localized the molecular determinants of potentiation by mutagenesis. Whole cell currents through TRPC5 were reversibly potentiated by micromolar concentrations of La\(^{3+}\), whereas millimolar concentrations were inhibitory. By comparison, TRPC6 was blocked to a similar extent by La\(^{3+}\) or Gd\(^{3+}\) at micromolar concentrations and showed no potentiation. Dual effects of lanthanides on TRPC5 were also observed in outside-out patches. Even at micromolar concentrations, the single channel conductance was reduced by La\(^{3+}\), but reduction in conductance was accompanied by a dramatic increase in channel open probability, leading to larger integral currents. Neutralization of the negatively charged amino acids Glu\(^{543}\) and Glu\(^{595/598}\), situated close to the extracellular mouth of the channel pore, resulted in a loss of potentiation, and, for Glu\(^{595}\)/Glu\(^{598}\) in a modification of channel inhibition. We conclude that in the micromolar range, the lanthanide ions La\(^{3+}\) and Gd\(^{3+}\) have opposite effects on whole cell currents through TRPC5 and TRPC6 channels. The potentiation of TRPC4 and TRPC5 by micromolar La\(^{3+}\) at extracellular sites close to the pore mouth is a promising tool for identifying the involvement of these isoforms in receptor-operated cation conductances of native cells.

Mammalian isoforms of the classical transient receptor potential channel (TRPC)\(^{3}\) subfamily, TRPC1–7, are likely candidates for cation channels mediating phospholipase C-depend-
Ca²⁺-permeable channels. Interestingly, recent studies have reported that 100 μM La³⁺ has potentiating effects on mouse, rat, and human TRPC4 and mouse TRPC5 (15, 16, 31). However, the actions of La³⁺ on TRPC4 and TRPC5 have not been characterized further. By contrast, other TRPCs are inhibited by micromolar concentrations of La³⁺ or Gd³⁺ (6, 29, 37–40).

Because the different effects of lanthanides are potentially an important distinguishing feature of the group 4 TRPC channels, we characterized the effect of these ions on TRPC5 in detail and compared them with those on TRPC6, a member of group 3. For this study we chose the rat TRPC6B slice variant (12), which lacks 54 amino acids at the distal N terminus compared with rat TRPC6A, and has not previously been characterized electrophysiologically. Unlike TRPC6A, TRPC6B has not been reported to be activated by agonist application but not by 1-oleyl-2-acetyl-sn-glycerol (OAG) (12). In whole cell patch clamp recordings, we found that TRPC5 was bimodally modulated by lanthanides, with potentiation at micromolar concentrations being succeeded by inhibition at millimolar concentrations. In contrast, TRPC6 was inhibited by micromolar concentrations and showed no potentiation. At the single channel level, the effects of La³⁺ on TRPC5 are complex, affecting the single channel conductance, the mean open time, and the frequency of channel openings. By site-specific neutralization of extracellular negatively charged amino acids, we have identified two sites, close to the pore mouth, that are involved in potentiation of TRPC5 by La³⁺.

EXPERIMENTAL PROCEDURES

Molecular Biology and Stable Transfection—The isolation of TRPC5 from mouse brain total RNA has been described previously (15). For cloning of TRPC6, total RNA was prepared from rat brain or A7r5 smooth muscle cells using a Trizol reagent (Invitrogen) according to the standard protocol. For cDNA synthesis, 1 μg of total RNA was reverse transcribed according to the protocol provided by the manufacturer using 200 units of Superscript II reverse transcriptase (Invitrogen) and 5 pmol of the primer 5′-CCAGTGAGCAGCAGAGTGACGAGGACTCGAGCTCAAGCTTATTTTTTTTTT (sense) and 5′-CCGATCCACCTATCTGCGGCTTTCCTCTTGTTT (antisense). The PCR products were subcloned into the pcRI.2 vector (Invitrogen) and the sequences confirmed by DNA sequencing of both strands (ABI Prism, PerkinElmer Life Sciences). The rat TRPC6 characterized in this study corresponds to the sequence of rTRPC6B published by Zhang and Saffen (12) (GenBank accession number AB051213) with the exception of two amino acid exchanges: M757I and S767F. The isolation of TRPC5 from human embryonic kidney (HEK293) cells (ATCC, Manassas, VA) were maintained according to the supplier’s recommendations. For transient transfection, cells were seeded in 25-mm culture dishes. The following day, 0.5–2 μg/dish of pcDNA3 vector containing the cDNA for TRPC5, TRPC5-YFP, or point mutants of TRPC5-YFP was mixed with 100 ng/dish of the rat histamine H₄ receptor (in pcDNA3) and, in the case of TRPC5, 50–100 ng/dish of pEGFP-C1 (Clontech), and transfected into the cells using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol. After 18–24 h, the cells were transfected and seeded onto glass coverslips. The T-REx and T-REx cells stably transfected with TRPC6 (T-REx-r6) were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 4 mM l-glutamine (Fluka, Taufkirchen, Germany), 100 units/ml penicillin, and 100 μg/ml streptomycin (both from Biochrom, Berlin, Germany). For T-REx-r6 cells, 5 μg/ml blasticidin (Invitrogen) and 250 μg/ml Zeocin were added to the culture medium. For some experiments, cells were transiently transfected with 100 ng/dish rat H₄ receptor in pcDNA3 and 50 ng of pEGFP-C1 (Clontech) according to the same protocol used for transient transfection of HEK293 cells.

For experiments on excised patches, coverslips were coated with poly-L-lysine. 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 1 μg/ml tetracycline (Roche Molecular Biochemicals).

Patch Clamp Recordings—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 had resistances of 2–6 megs (whole cell recordings) or 6–16 megs (single channel recordings) when filled with the standard intracellular solutions.

Whole cell recordings were performed as described previously (15, 31). To quantify current potentiation and current inhibition observed by bath application of lanthanides, we interpolated currents before and after application of lanthanides and normalized the values obtained in the presence of the lanthanides to the interpolated values. Interpolation was done to avoid errors arising from the fact that TRPC5 and TRPC6 currents decay with time. All current amplitudes were calculated as the difference between resting and histamine-, OAG-, or AIP₃-induced current levels. The concentration-response curve obtained for lanthanum inhibition of TRPC6 currents was fitted with Equation 1 to determine the IC₅₀ value. Values for the relative Ca²⁺-permeability (Pᵣ/Pₕ) were calculated from Equation 2.

$$P_{C/FA} = \frac{[F]}{[A]} \times (\exp(\frac{F}{RT})/V_{Ca} - V_{Na} ) \times (1 + \exp(\frac{-F}{RT}))$$

where V_{Ca} and V_{Na} are the reversal potentials in external solutions containing Ca²⁺ and Na⁺, respectively, and R, T, and F have their usual meanings.

For single channel recordings, the standard excised outside-out patch configuration (41) was used. After filtering at 10 kHz, single channel data were initially recorded onto digital audiotape (DAT, Biologic, Claix, France). For offline analysis, the single channel data were filtered at 1 kHz, subsequently digitized at 15 kHz, and analyzed with the Biopac software (Advanced Instruments, Foster City, CA). Single channel amplitudes were obtained from events with open durations of more than 2 ms. In the case of TRPC5, channel activity was expressed as N_P, the product of the minimum number (N) of channels in the patch (obtained from the observed number of open levels) and the open probability (Pₒ). N_P 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 La³⁺, we used an algorithm established by Fenwick et al. (42) to obtain a reliable estimate of mean open times in the absence and presence of La³⁺. The general applicability of the algorithm has been confirmed (43). The overall estimate of mean channel open time (tₒ) was calculated according to tₒ = Σ(t_i*N_i)/N, where N is the number of all channel openings (transitions between a given level j and a subsequent level j + 1), t_i designates the dwell time of a given level j, and the sum extends over all levels encountered in the recording. Values for tₒ were extracted from idealized traces generated with pcamps.

The standard extracellular solution contained 140 mM NaCl, 5 mM CaCl₂, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES (pH 7.4 with NaOH). For NMDG⁺ solutions, Na⁺ and Ca²⁺ were replaced by N-methyl-D-glucamine (NMDG), and Ca²⁺ was omitted. In solutions with 20 mM CaCl₂, the NaCl or NMDG⁺ concentration was reduced to 115 mM. The standard intracellular solution contained 110 mM cesium methanesulfonate, 25 mM CsCl, 2 mM MgCl₂, 3.92 mM CaCl₂, 10 mM EGTA, and 30 mM HEPES (pH 7.2 with CsOH) with a calculated [Ca²⁺] of 100 mM. In some experiments, a pipette solution with stronger Ca²⁺-buffering (30 mM BAPTA) with a calculated [Ca²⁺] of 100 mM was used. It contained 50 mM cesium methanesulfonate, 25 mM CsCl, 2 mM MgCl₂,
Lanthanide Potentiation of TRPC5

Receptor-activated Currents through TRPC5 and TRPC6—We first performed whole cell voltage clamp recordings to compare currents mediated by TRPC5 with those mediated by TRPC6. The latter isoform was the rat TRPC6B splice variant, which has, to date, only been studied in fluorometric experiments (12) and has not been characterized electrophysiologically. As described previously (15), cells expressing TRPC5 and the histamine H1 receptor displayed spontaneous channel activity. By contrast, no constitutive activity was observed at a holding potential of −60 mV in the T-REx-r6 cell line 24–48 h after induction with tetracycline. Currents on break-in were in the range of −0.1 to −1.87 pA/pico farads (n = 24), values not significantly different from those in control cells. Characteristic currents were activated by the application of 100 μM histamine or infusion of AIF4+, a direct activator of G-proteins (44) in TRPC5- (Fig. 1A) or TRPC6-expressing cells but not in control cells. Furthermore, TRPC6-mediated inward currents could also be elicited by adding the membrane-permeable diacylglycerol OAG (100 μM, n = 11) (Fig. 1B) to the bath solution. This finding is in contrast to those of Zhang and Saffen (12), who detected receptor- but not OAG-induced Ba2+ influx in COS cells expressing the species and splice variant used here.

As reported previously for murine and human isoforms of TRPC5 and TRPC6 (9, 15), the I-V relation of currents mediated by both TRPC5 and TRPC6 displayed a characteristic doubly rectifying shape and reversal potentials close to 0 mV, indicative of poor cation selectivity (Fig. 1, C and D). Although the I-V relations of currents mediated by TRPC5 and TRPC6 were similar, currents in TRPC6-expressing cells display a slightly stronger outward rectification.

Effects of Lanthanides on Currents Mediated by TRPC5 or TRPC6—We compared the effects of lanthanides on currents mediated by TRPC5 and TRPC6. When 10 μM La3+ was applied to HEK293 cells coexpressing mTRPC5 and the histamine H1 receptor during exposure to 100 μM histamine, inward and outward currents were increased (Fig. 1A). Comparison of the I-V relations before and after application of La3+ revealed that the potentiating effect of La3+ was much more pronounced at negative potentials (Fig. 1C). The effectiveness of very low concentrations of La3+ and the absence of a shift in reversal potential exclude the possibility that La3+ acts as a charge carrier for the additional inward current. In control cells transfected only with the histamine H1 receptor, neither application of histamine nor subsequent addition of La3+ resulted in a current increase (n = 6). In these cells, application of La3+ decreased basal leak currents rather than causing potentiation. These data suggest that La3+ affects currents carried by TRPC5. By contrast, when 100 μM La3+ was applied to TRPC6-expressing T-REx-r6 cells stimulated with 100 μM OAG, rapid inhibition of the inward current was observed at a holding potential of −60 mV (Fig. 1B). Inhibition was almost complete at all potentials tested (Fig. 1, B and D). For both TRPC5- and TRPC6-mediated currents, the effects of lanthanides were readily reversible on wash-out (Fig. 1, A and B).

The concentration dependence of the effects of lanthanides on TRPC5 has not been studied previously. We therefore tested the effect of various concentrations of La3+ or Gd3+ on TRPC5-mediated currents and compared them with those on TRPC6 (Fig. 2, A and B). Because agonist-induced currents in T-REx-r6 cells were relatively short lived, infusion of AlF4− via the pipette was preferred. AlF4−-induced currents in T-REx-r6 cells were indistinguishable from agonist-induced currents with respect to the I-V relation and their sensitivity to lanthanides. However, the current response was slowed considerably, thus allowing the effects of different concentrations of lanthanides to be investigated in the same cell. The pooled concentration-response relationships for TRPC5- and TRPC6-mediated currents are shown in Fig. 2, C and D. Relative inhibition or potentiation was calculated as described under “Experimental Procedures.” The inhibitory effect of lanthanides on TRPC6 began at concentrations around 1 μM, and application of 1 μM resulted in near complete inhibition. The concentration-inhibition relationship for La3+ inhibition of TRPC6 was sigmoidal (Fig. 2D) with an IC50 value of 6.1 μM. The effects of Gd3+ on currents mediated by TRPC6 were examined at two concentrations, 10 μM and 1 mM. The values for relative inhibition by Gd3+ were similar to those obtained with La3+. As seen in Fig. 2, A and C, the effects of La3+ and Gd3+ on currents mediated by TRPC5 were more complex. Starting at a concentration of around 1 μM, both La3+ and Gd3+ increased TRPC5-mediated currents in a concentration-dependent manner. The largest potentiation was observed between 10 μM and 1 mM and resulted, on average, in a 3-fold...
increase in current. At millimolar concentrations, the potentiating effect was reduced. In 5 mM La^{3+}, the mean current was less than the control value, indicative of inhibition. In contrast, in 5 mM Gd^{3+}, the mean relative current amplitude was \(-120\%\). It has to be noted, however, that the effects of 5 mM Gd^{3+} on TRPC5-mediated currents were not uniform. Some cells responded to this concentration with potentiation of currents (n = 2/5), whereas others responded with current inhibition (n = 3/5). Similarly, dual effects of lanthanides were often observed during wash-in or wash-out of the ions at a concentration of 5 mM. On wash-in, inhibition was sometimes preceded by transient potentiation and on wash-out, removal of inhibition followed by transient potentiation (see inset in Fig. 2A). Thus, lanthanides inhibit TRPC6 but have dual effects on TRPC5. Low concentrations potentiate TRPC5 currents, but high concentrations are less effective and may result in inhibition.

**Effect of Extracellular Ca^{2+} on Potentiation by La^{3+}**—To investigate whether physiological cations can act at the same site as lanthanides, we tested the effect of Ca^{2+}. Raising [Ca^{2+}] from 2 to 20 mM resulted in a rapid increase in TRPC5 channel currents similar to that produced by micromolar La^{3+} concentrations (n = 5; data not shown). This similarity includes a stronger potentiation of currents at negative than at positive potentials. In addition to the rapid increase in inward current, there was a slower increase in both inward and outward currents which may reflect channel activation by an increase in [Ca^{2+}], resulting from Ca^{2+} entry through the channel (14, 15).

The decay of current upon returning [Ca^{2+}], to 2 mM was slow compared with that after removal of La^{3+}. In the presence of 20 mM Ca^{2+}, potentiation by 10 mM La^{3+} was prevented or strongly reduced (n = 5). These results indicate that Ca^{2+} can compete with La^{3+} for the same site.

**High Concentrations of Intracellular BAPTA Do Not Reduce Potentiation by La^{3+}**—In general, the actions of lanthanides on ion channel behavior are considered to be strictly confined to the extracellular face of the plasma membrane. However, a recent report on lanthanide-induced inhibition of currents mediated by human TRPC3 transiently expressed in Chinese hamster ovary cells suggested intracellular regulatory actions for both La^{3+} and Gd^{3+}, although additional extracellular effects could not be excluded (39). If the effects of La^{3+} on TRPC6- and TRPC5-mediated currents result from an intracellular action of the free cation they should depend on the trivalent cation buffering capacity of the intracellular solution. Thus, raising the buffer capacity of the intracellular solution by replacing 10 mM EGTA with 30 mM BAPTA, which was used in previous experiments. B, inhibitory effect of La^{3+} on TRPC6 currents recorded with a 30 mM BAPTA-buffered pipette solution. Currents at \(-60\) mV were elicited by 100 mM histamine in TRPC6-expressing T-REx cells. C and D, I-V relationships in the presence and absence of La^{3+} obtained from voltage ramps from \(-100\) to +100 mV in the experiments in A and B, respectively.

The decay of current upon returning [Ca^{2+}], to 2 mM was slow compared with that after removal of La^{3+}. In the presence of 20 mM Ca^{2+}, potentiation by 10 mM La^{3+} was prevented or strongly reduced (n = 5). These results indicate that Ca^{2+} can compete with La^{3+} for the same site.
yielded a single channel chord conductance at \(-60\) mV of 46.6 ± 1.5 picoSiemens (n = 12).

When La\(^{3+}\) was added to the bath solution at a concentration of 100 \(\mu\)M, the open probability of TRPC5 was dramatically increased (Fig. 5A). At the same time, the single channel current amplitude was approximately halved compared with that recorded before the addition of La\(^{3+}\) (Fig. 5A). An analysis of the La\(^{3+}\) block of TRPC6 was precluded by the extremely short events, which also made estimates of open probability difficult. Channel activity was, however, abolished by the addition of 100 \(\mu\)M La\(^{3+}\) (Fig. 5B) and was associated with a decrease in the frequency of channel openings (Fig. 5C).

A reduction in the single channel current of TRPC5 upon application of 100 \(\mu\)M La\(^{3+}\) was observed at all potentials (Fig. 6, A and B), with no increase in open channel noise. This result indicates that La\(^{3+}\) may produce a very fast, flickery block of the channel, not resolvable under our recording conditions, at a site outside the membrane electrical field. Inhibition by an allosteric mechanism cannot be excluded. The shape of the i-V relationship of TRPC5-mediated currents and the reversal potential of around 0 mV were preserved in the presence of La\(^{3+}\) (Fig. 6B). The effects of different La\(^{3+}\) concentrations on single channel amplitude are shown in Fig. 6C. Increasing the La\(^{3+}\) concentration from 1 \(\mu\)M to 5 mM resulted in a successive reduction of single channel current. The effects of different La\(^{3+}\) concentrations on the single channel properties including single channel current amplitude, open probability, mean open time, and opening frequency are summarized in Table I. The open probability was roughly doubled in the presence of 1 \(\mu\)M La\(^{3+}\) and increased about 10-fold in the presence of 100 \(\mu\)M La\(^{3+}\). The increased open probability of TRPC5 channels in the presence of La\(^{3+}\) was the result of both increased open times and higher frequency of channel openings (Table I). Because brief events (<0.5 ms, see “Experimental Procedures”) were excluded from the analysis, and the proportion of these decreases with increasing La\(^{3+}\) concentrations, the effect on mean open time will be underestimated. The combined effect of the increase in NPo and the decrease in single channel current was an ~10-fold increase in the total current through the patch (NPo \(\times\) i). Interestingly, the increase in patch current had a nearly identical dependence on the La\(^{3+}\) concentration to the whole cell current. From the single channel data, the EC\(_{50}\) for potentiation by La\(^{3+}\) was around 3 \(\mu\)M, whereas a 50% reduction of the single channel current occurred at a La\(^{3+}\) concentration of 100 \(\mu\)M. Taken together, the above data suggest that La\(^{3+}\) exerts diverse effects on single channel activity, having opposite effects on channel conductance and open probability.

Identification of Amino Acids Involved in Potentiation of TRPC5 by La\(^{3+}\)—In an attempt to identify the site involved in potentiation of TRPC5 by La\(^{3+}\), we searched for negatively charged amino acids (Glu and Asp residues) in the putative extracellular loops of the TRPC5 protein. We identified 10 residues that are conserved in TRPC5 (Fig. 7A) and TRPC4 and generated six point mutants in which individual residues, and two in which pairs of close neighbors, were neutralized (Glu to Gln, or Asp to Asn). The C-terminally YFP-tagged mutants were expressed in HEK293 cells and their subcellular distribution and membrane targeting determined by confocal laser microscopy. Like wild type TRPC5 (TRPC5-wt) (15), the mutants displayed a clustered appearance in the plasma membrane and retention in a perinuclear compartment.

As an initial probe for channel function, we tested for the presence of histamine-induced Mn\(^{2+}\) influx in fura-2-AM-loaded cells. Five of the mutants (D392N, E404Q and E543Q, E570Q and E595Q/E598Q) displayed robust accelerations in Mn\(^{2+}\) influx upon addition of histamine, E559Q only weak responses.
TABLE I

Effect of different La$^{3+}$ concentrations on the single channel properties of heterologously expressed TRPC5

| Concentration (M) | Current (pA) | NPo | Mean open time (ms) | Frequency (Hz) | N 
|-------------------|-------------|-----|---------------------|----------------|------
| -60 mV/HA         | -2.48 ± 0.07| 12  | 0.06 ± 0.03         | 7.5 ± 2.8      | 8    
| +La$^{3+}$ (1 μM) | +1.85 ± 0.03| 4   | 0.15 ± 0.06         | 9.2 ± 2.2      | 3    
| +La$^{3+}$ (10 μM) | +1.44 ± 0.03| 5   | 0.94 ± 0.32         | 27.4 ± 7.5     | 3    
| +La$^{3+}$ (100 μM) | +1.31 ± 0.03| 8   | 1.13 ± 0.65         | 41.2 ± 19.4    | 5    
| +La$^{3+}$ (1 mM) | +1.17      | 1   | 1.09                | 23.4           | ND   
| +La$^{3+}$ (5 mM) | -0.74 ± 0.02| 3   | ND                  | ND             | ND   

(data not shown). In contrast, E467Q/E470Q and E479Q did not respond. In whole cell recordings, the mutants that showed robust accelerations in Mn$^{2+}$ influx displayed histamine-activated currents indistinguishable from those of TRPC5-wt (n ≥ 10 for each mutant). Addition of La$^{3+}$ resulted in increases in current in the mutants D392N, E404Q, and E570Q similar to those observed for TRPC5-wt (data not shown). In contrast, neutralization of negatively charged amino acids at two sites in the putative pore-forming loop, E543Q, just after transmembrane segment S5, and E595Q/E598Q, close to S6, led to a loss of La$^{3+}$-induced potentiation (Figs. 7 and 8). Both of these mutants did, however, display inhibition. This inhibition was different for the two mutants. Both inward and outward currents mediated by the mutant E543Q were inhibited to a similar extent by La$^{3+}$ (Fig. 7, B and C). In contrast, inward currents mediated by the mutant channel E595Q/E598Q were inhibited to a larger extent than outward currents (Fig. 8, A and B).

We also tested whether potentiation by Ca$^{2+}$ was influenced in the mutants E543Q and E595Q/E598Q. Currents through the mutants did not show the rapid potentiation observed upon raising [Ca$^{2+}$], from 2 to 20 mM for TRPC5-wt (data not shown). Both inward and outward currents mediated by
E543Q were inhibited in 20 mM Ca\(^{2+}\) (n = 3, data not shown). In contrast, currents through E595Q/E598Q slowly increased to a maximum, then declined spontaneously in the continued presence of 20 mM Ca\(^{2+}\) (n = 3, data not shown). In these experiments, there was a more noticeable shift in the current reversal potential on raising [Ca\(^{2+}\)]\(_o\), from 2 to 20 mM for E595Q/E598Q than for TRPC5-wt or E543Q. We therefore quantified the relative Ca\(^{2+}\) permeability of E595Q/E598Q and compared it with that of TRPC5-wt. Current reversal potentials were first measured from voltage ramps in a nominally Ca\(^{2+}\)-free, Na\(^+\) solution, then in a Na\(^+\)-free (NMDG\(^-\)) solution containing 20 mM Ca\(^{2+}\). From the reversal potentials, we calculated values for \(P_{Ca}\)/\(P_{Na}\) of 1.83 ± 0.18 (n = 3) for TRPC5-wt, a value close to the 1.79 in our previous study (15), and 4.28 ± 0.08 (n = 4) for E595Q/E598Q.

Thus, the mutants E543Q and E595Q/E598Q did not show rapid potentiation by La\(^{3+}\) and Ca\(^{2+}\), further supporting a similar site of action. Furthermore, mutant E595Q/E598Q had a higher relative Ca\(^{2+}\) permeability than TRPC5-wt.

**Effects of La\(^{3+}\) on the Single Channel Properties of the Mutants E543Q and E595Q/E598Q**—In outside-out patches, the single channel properties of the mutant E543Q were similar to those of TRPC5-wt (Fig. 7D). The mean single channel current at −60 mV was −2.63 ± 0.10 pA (chord conductance, 43.8 picosiemens; n = 4), a value not significantly different (p = 0.28) from that of the wild type channel. It is noteworthy that very high levels of channel activity were observed in patches from mutant E543Q-expressing cells, necessitating the use of much smaller pipettes. Addition of 100 μM La\(^{3+}\) to the extracellular solution reduced the current amplitude to −1.47 ± 0.16 pA (Fig. 7D), a decrease similar to that observed for TRPC5-wt. Like the inhibition of TRPC5-wt, the reduction in channel current was not accompanied by an increase in open channel noise. However, in contrast to the wild type channel, application of 100 μM La\(^{3+}\) did not result in an increase in \(N_{Po}\) (0.50 ± 0.21 and 0.53 ± 0.14 (n = 4; p = 0.8) in control and 100 mM La\(^{3+}\), respectively). The mutant E595Q/E598Q showed more drastic changes in its single channel properties (Fig. 8C). The single channel current of −2.98 ± 0.03 pA at −60 mV (chord conductance, 49.7 picosiemens; n = 9) was significantly (p < 0.01) larger than that for TRPC5-wt. Application of 100 μM or 1 mM La\(^{3+}\) resulted in weaker reductions in current amplitude than in TRPC5-wt (−2.50 ± 0.03 pA, n = 7 and −1.25 ± 0.07 pA, n = 4, respectively) and a clear increase in open channel noise indicative of a slower, flickery channel block (Fig. 8C).
Thus, at the single channel level, neither mutant showed potentiation. E543Q had a similar conductance and was inhibited by La$^{3+}$ in a manner similar to TRPC5-wt. In contrast, E595Q/E598Q had a higher conductance than TRPC5-wt or E543Q, and inhibition by La$^{3+}$ was modified.

**DISCUSSION**

In the present study, we show that currents mediated by TRPC5 and TRPC6 are affected differently by the lanthanides La$^{3+}$ and Gd$^{3+}$. Although whole cell currents through TRPC6 were inhibited concentration-dependently by lanthanides, those through TRPC5 were potentiated by low concentrations but inhibited by high concentrations. The dual effect of La$^{3+}$ on TRPC5 was also observed at the single channel level and involved a combination of an inhibitory effect on channel amplitude and an increase in channel open probability. By analysis of point mutants, we identified two sites, close to the extracellular mouth of the pore, which are involved in La$^{3+}$- and Ca$^{2+}$-induced channel potentiation.

Inhibitory effects of different di- and trivalent cations, including La$^{3+}$ and Gd$^{3+}$, have been described for most Ca$^{2+}$-permeable channels. Accordingly, current block by bath application of lanthanide ions has been reported for several members of the TRPC subfamily of TRP channels, e.g. for human and mouse TRPC3 (29, 37–39), for mouse TRPC6 (6, 45), and for human TRPC7 (40). There is considerable variability in the IC$_{50}$ values obtained, with higher values in Ca$^{2+}$ imaging experiments than in electrophysiological recordings. The IC$_{50}$ value obtained for rat TRPC6 in the present study was in good agreement with the values obtained in whole cell patch clamp experiments for mouse TRPC6 (6) and human TRPC3 (39).

The pronounced increase in agonist-induced currents in TRPC5-expressing cells with micromolar La$^{3+}$ in the present study are in agreement with previous studies on this channel (15, 16), and we have extended this observation to Gd$^{3+}$, which is approximately equally effective. A further novel finding of the present study is that higher lanthanide concentrations (≥1 mM) were less effective in potentiating the current and even reversibly inhibited currents carried by TRPC5. Millimolar concentrations of Ca$^{2+}$ also potentiated agonist-induced currents and prevented the effects of micromolar La$^{3+}$, suggesting that physiological divalent cations may also bind at the same site.

Evidence for two different actions of lanthanides on the channel was supported by the effects of La$^{3+}$ on single channel currents in outside-out patches. La$^{3+}$ caused a concentration-dependent decrease in single channel current amplitude, while, at the same time, increasing the channel open probability ($N_P$). Both effects were already observed at a concentration of 1 μM. The concentration dependence of the increase in current in outside-out patches ($N_P$,t) closely paralleled the increase in whole cell current, although the maximum potentiation was, on average, about 3–4-fold higher in outside-out patches than in whole cell experiments. Because of our inability to resolve currents at millimolar concentrations of La$^{3+}$, it is not clear from the single channel data why potentiation declines and inhibition occurs. A decrease in single channel current is at least partly responsible for the decrease in whole cell current.

There are few reports of potentiating effects of La$^{3+}$ on ion channel currents, and more importantly, to our knowledge, there are no reports that describe dual effects on ion channel activity. Potentiating actions of 100 μM La$^{3+}$ have been observed in whole cell recordings for mouse, rat, and human TRPC4- and mouse TRPC5-mediated currents (15, 16, 31) and for receptor-operated currents in cells expressing mouse TRPC1 and mouse TRPC5 (16). In the latter, heteromultimers of TRPC1 and TRPC5 are thought to be formed, which, compared with homomeric TRPC5, have a drastically reduced single channel current (~0.5 pA at a holding potential of ~60 mV). The single channel current amplitude was not affected by the inclusion of La$^{3+}$ in the pipette solution. For native nonselective cation currents, there is one report of a potentiation of the native current ($I_{\text{Na}}$) in rat illeal smooth muscle cells by La$^{3+}$, with an apparent $K_d$ of 190 μM (46). From relaxation analysis, prolonged single channel mean open life times were suggested to be the main cause of the augmentative effect of La$^{3+}$. Interestingly, in the mouse, TRPC4 is expressed in this tissue (47). With regard to heterologously expressed TRPC4 and TRPC5, it should be noted that some studies reported an inhibition by micromolar lanthanide concentrations (14, 48).

The loss of the potentiating effects of La$^{3+}$ and Ca$^{2+}$ in mutants of two sites (Glu$^{543}$ and Glu$^{595}$/Glu$^{598}$), which, according to models of TRP channel structure, are located opposite each other at the start and end of the pore-forming loop between S5 and S6, strongly supports an extracellular site of action. Importantly, identical amino acids are present in TRPC4 at the positions corresponding to Glu$^{543}$ and Glu$^{595}$ in TRPC5, but acid amino acids are not present at corresponding positions in TRPC3, TRPC6, and TRPC7. Larger variations in structure prevent an identification of corresponding residues in TRPC1. The differences between the TRPC isoforms provide an explanation for the specificity of the potentiating effect for TRPC4 and TRPC5. Interestingly, these sites are analogous to those in TRPV1 (VR1) which are involved in proton-mediated channel potentiation (Glu$^{600}$) and proton-mediated channel activation (Glu$^{648}$) (49) and can modulate sensitivity to the activator capsaicin (49, 50). Indeed, at the latter site TRPC4, TRPC5, and TRPV1 have identical EFTXE motifs. Because of the distal steps leading to activation of this channel and the activation mechanism are not known, it is not clear how La$^{3+}$ or Ca$^{2+}$ binding to the extracellular sites results in current potentiation. By analogy to TRPV1, where neutralization of Glu$^{600}$ and Glu$^{648}$ leads to potentiation of the capsaicin sensitivity (49, 50), it is tempting to speculate that La$^{3+}$ or Ca$^{2+}$, by neutralizing the negative charges, potentiate the response of TRPC5 to its unknown activator.

The TRPC5 mutation E595Q/E598Q also affected inhibition by La$^{3+}$, whereas E543Q did not. For the wild type channel, the reduction in single channel current by La$^{3+}$ at all potentials without an increase in open channel noise is indicative of a fast block at a site outside the membrane electrical field. Similarly, inhibition of whole cell currents in the mutant E543Q, which lacked potentiation, was potential-independent. In contrast, the mutant E595Q/E598Q showed a slower flickery block at the single channel level and a clear potential dependence of whole cell current inhibition, with inward currents being more strongly reduced than outward currents. The loss of the fast block by mutation at the extracellular site E595Q/E598Q and the potential dependence of the block remaining after mutation indicate that in both cases La$^{3+}$ blocks the channel from the outside. The effect of this mutation on inhibition by La$^{3+}$, the increase in single channel current, and the increase in $P_{\text{cat}}$/Na suggest that this site lies close to, or in, the permeation pathway. Considering the change in channel inhibition by La$^{3+}$, it is possible that the increase in single channel current in E595Q/E598Q results from a reduction in block by a physiological cation. By analogy to other channels with similar structure, the glutamates will form a negatively charged ring around the extracellular pore mouth, with, in tetramers, at least 12 negatively charged residues. These amino acids may act as “gatekeepers” controlling cation entry into the pore.

Further evidence that both potentiation of TRPC5 and inhi-
bition of TRPC6 by lanthanides results from an extracellular action of La$^{3+}$ is provided by the presence of the effects in experiments with intracellular EGTA buffers and their persistence in the presence of higher concentrations of BAPTA. Both buffers have a very high affinity for lanthanides. Recently, Halaszovich et al. (39) suggested that La$^{3+}$ and Gd$^{3+}$ block human TRPC3 channels from the cytosolic side of the membrane and that different apparent IC$_{50}$ values might simply reflect different uptake rates for lanthanide ions in different cell types (see below). Our data support an extracellular site of action on TRPC6 and on TRPC5, although we cannot exclude additional intracellular effects.

Because of the variability in results from different laboratories, the applicability of results from heterologous overexpression studies on TRPC channels to native channels has recently been questioned (e.g. 5). However, for TRPC6, at least, properties nearly identical to those observed after overexpression are seen for native channels in vascular smooth muscle cells (6, 7). The activation of TRPC3, TRPC6, and TRPC7 by DAGs in a protein kinase C-independent manner is a characteristic feature. Another characteristic property is the shape of the I-V relation, with a strong reduction in current at high voltages, a feature shared with TRPC4 but not with most other TRPC channels. A previous report that the store depletion-independent activation, as well as stimulation by ITP, is provided by the presence of the effects in native cells. TRPC4 and TRPC5 (15, 16, 31) stand out in this respect, the applicability of results from heterologous overexpression studies on TRPC channels to native cells has recently been questioned (e.g. 5). However, for TRPC6, at least, properties nearly identical to those observed after overexpression are seen for native channels in vascular smooth muscle cells (6, 7).

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