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Substance P Evokes Cation Currents Through TRP Channels in HEK293 Cells

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Oh, E. J., T. D. Gover, R. Cordoba-Rodriguez, and D. Weinreich. Substance P evokes cation currents through TRP channels in HEK293 cells. *J Neurophysiol* 90: 2069–2073, 2003; 10.1152/jn.00026.2003. Activation of any of the three known tachykinin receptors (NK1R, -2R, or -3R) can cause a rise in $[Ca^{2+}]_i$ via a pertussis toxin-insensitive heterotrimeric G protein, G_q/G_{11} , activation of phospholipase C (PLC), and a membrane depolarization. Tachykinins can depolarize neurons by two distinct mechanisms: 1) they reduce a resting K^+ current in many neurons or 2) in parasympathetic and vagal primary sensory neurons, they activate a nonspecific cation current (I_{cat}). Transient receptor potential channels (TRPC) are nonspecific cation channels that can be activated by a rise in $[Ca^{2+}]_i$ in a PLC-dependent manner. The present work tests whether NK2R can signal TRPC. We applied standard whole cell patch-clamp recordings to HEK293 cells stably transfected with the human TRP3 channels (TRP3C), and transiently transfected with a functional NK2R-EGFP. Bath applied Substance P (SP, 1 μ M) induced an I_{cat} in the cells expressing both TRP3C and NK2R. I_{cat} reached its peak value in approximately 3 min (195 ± 120.0 s, mean \pm SE, $n = 20$), had a peak density of 11.3 ± 3.48 pA/pF ($n = 24$), and was blocked by an NK2R-specific antagonist (SR48968, 100 nM). The E_{rev} value for the SP current was 6.8 ± 7.66 mV ($n = 6$), suggestive of a nonspecific cation channel. I_{cat} was not measurable in TRP3C-expressing HEK293 cells without NK2R expression ($n = 6$) or in wild-type HEK293 cells with NK2R expression ($n = 12$). These data indicate that NK2R can be functionally coupled to TRP channels in HEK293 cells and suggest that SP-induced cation currents in vagal primary sensory neurons might be mediated by TRPC.

INTRODUCTION

Substance P (SP) can depolarize neuronal membranes by two distinct mechanisms. In most neurons, SP decreases resting K^+ conductances (Nakajima and Nakajima 1994). In guinea pig vagal afferent neurons and rat dorsal root ganglion neurons, SP depolarizes the membrane by increasing a cation conductance (Inoue et al. 1995; Weinreich et al. 1997). The three known tachykinin receptors (NK1R, NK2R, and NK3R) are each coupled to G proteins, which are pertussis toxin-insensitive (G_q/G_{11}), and linked to the inositol phospholipid hydrolysis system (Khawaja and Rogers 1996; Otsuka and Yoshioka 1993). When SP binds to its receptor, phospholipase C (PLC) can be activated via the action of G proteins and active PLC leads to the production of inositol triphosphate

(IP₃) and diacylglycerol (DAG). However, it is not yet known how these second messenger systems activate cation conductances, nor is the nature of channels opened by tachykinin receptor activation.

TRP channels (TRPC) are nonselective cation channels that are permeable to monovalent and divalent ions including Ca^{2+} (Clampham et al. 2001). Certain ligands binding to plasma membrane receptors will activate a phospholipase C (PLC) pathway via G-proteins and generate IP₃ and DAG. This pathway is known to activate several types of TRPC. However, there is still little information about the role of these channels and their endogenous ligands. Several ligands that lead to the activation of TRPC have been recently reported including bradykinin (Delmas et al. 2002), BDNF (Li et al. 1999), and catecholamine (Inoue et al. 2001). Because tachykinin receptors are G protein-coupled and they are coupled to nonselective cation channels, TRPC may be a final effector of activated tachykinin receptors.

In nodose ganglion neurons (NGNs) of guinea pigs, SP depolarizes the membrane potential with an associated increase in the membrane conductance and a reversal potential (E_{rev}) value near -27 mV, suggesting that a nonspecific cation conductance may mediate the SP response (Moore et al. 1999). These results are compatible with the hypothesis that TRPC may be a final target of activated tachykinin receptors. To evaluate this surmise, we applied the whole cell patch-clamp recording technique, microfluorometry, and immunocytochemical measurements to HEK293 cells, stably transfected with the human TRP3 channel (TRP3C) and transiently transfected with a rat NK2R.

In HEK293 cells expressing both TRP3C and NK2R, our results reveal that SP can induce inward currents reversing in polarity at approximately $+7$ mV and an increase in $[Ca^{2+}]_i$. These data reveal that activation of tachykinin receptors can be functionally coupled to TRPC.

METHODS

Cell cultures and transfection

HEK293 cells stably transfected with HA-tagged human TRP3 channels (TRP3C) were obtained from Dr. Donald Gill (University of Maryland, Baltimore, MD). Stably transfected cells and wild-type

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HEK293 cells were maintained at 37°C and 5% CO₂ in Dulbecco's modified eagle's medium (DMEM, Life Technologies, Rockville, MD) with 10% heat inactivated fetal bovine serum (FBS, Life Technologies), 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Sigma, St. Louis, MO). In addition, the medium for the stably transfected cells contained 400 µg/ml G418 (Biosource International, Camarillo, CA). Both cell lines were split twice a week. For transfection and electrophysiological study, cells were plated on 25-mm coverslips coated with poly-D-lysine (0.1 mg/ml, Sigma). NK2R-EGFP constructs ligated into the pCEP4 expression vector were obtained from Dr. Jean-Luc Galzi (CNRS, Illkirch, France). JM109 cells were transformed with the plasmid containing the NK2R-EGFP construct. HEK293, both wild-type and cells stably transfected with TRP3C, were transiently transfected with the purified plasmids using the calcium phosphate precipitation method (Paborsky et al. 1990). Cells were used for patch-clamp recording, intracellular calcium measurements, or immunohistochemistry 48 h after transfection.

Electrophysiology

The whole cell patch-clamp technique was employed with an Axopatch-1C amplifier and pCLAMP8 software (Axon Instruments, Union City, CA). Cells were identified as being successfully transfected with NK2R-EGFP by the presence of green fluorescence. Patch pipettes, borosilicate glass, 2–4 MΩ resistance, were fabricated using a Narishige pp-83 puller (Narishige, Tokyo, Japan). Pipettes were filled with solution composed of (in mM) 130 CsCl, 10 HEPES, 11 EGTA, 1 CaCl₂, 2 MgCl₂, 0.3 ATP, and 0.03 GTP; pH 7.3; 301 mOsm. CaCl₂ and MgCl₂ were purchased from J. T. Baker (Phillipsburg, NJ), and other chemicals were from Sigma. Coverslips were superfused (2–4 ml/min) with room temperature (22–23°C) Locke solution with a composition of (in mM) 136 NaCl, 5.6 KCl, 1.2 NaH₂PO₄, 14.3 NaHCO₃, 2.2 CaCl₂, 1.2 MgCl₂, and 10 glucose; pH 7.3; 320 mOsm. Except NaCl (Sigma), all the reagents were purchased from J. T. Baker. Criteria for cell inclusion were as follows: series resistance (R_s) < 10 MΩ, membrane input resistance (R_m) > 100 MΩ, and stable recording with 80% R_s compensation. Cells were held at –80 mV during current recordings. To measure conductance changes and reversal potential (E_{rev}) values, ramp voltage-clamp commands were performed repetitively (once every 3 s) during the entire time course of current change. With each ramp, cells were depolarized from –80 to +60 mV at 0.5 mV/ms. SP (Calbiochem, San Diego, CA), NKA (Bachem, King of Prussia, PA), and SR48968 (a gift from Zeneca, Wilmington, DE) were diluted in Locke solution to a working concentration of 1 µM from 10-mM stock solutions prepared in distilled water or DMSO and stored at –20°C. SKF96365 (30 µM, Calbiochem) was prepared similarly from a 30-mM stock solution.

Immunohistochemistry

Cells were fixed in phosphate-buffered saline (PBS) containing 4% sucrose and 4% paraformaldehyde (PF) for 20 min at 4°C. After two washes in PBS, cells were permeabilized with 0.1% Triton X-100 for 5 min and washed once with PBS. To rid the cells of remnant PF and control for autofluorescence, we treated the cells with PBS containing 0.1% sodium borohydride for 10 min. After rinsing cells three times with PBS, they were incubated in PBS containing 1% bovine serum albumin (BSA) and the primary antibody (mouse anti-HA, 1:400, Roche, Indianapolis, IN) for 1 h at room temperature. After rinsing cells three times with PBS, they were incubated in PBS containing Cy3-tagged goat anti-mouse IgG (1:500, Jackson Immunoresearch, West Grove, PA) for 45 min at room temperature. Cells were then washed three times with PBS and coverslipped with Mowiol (Calbiochem) as mounting medium. After 2 h, the mounting medium was hard, and coverslips were stored at –20°C prior to microscopic anal-

ysis. Cells were visualized using an Axiovert confocal microscope (LSM410, Zeiss, Oberkochen, Germany). Cells transfected with NK2R-EGFP were identified using a 488-nm laser for excitation and emission filter and dichroic appropriate for fluorescein. Cells transfected with TRP3C were visualized using a 568-nm laser for excitation and emission filter and dichroic appropriate for rhodamine to detect Cy3-tagged antibodies. Optical sections (1 µm thickness) across the middle section of the cells were taken and digitized using Zeiss software. Unless specified, all reagents were purchased from Sigma.

Intracellular calcium measurement

HEK293 cells were loaded with 1 µM fura-2 AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Cells expressing EGFP were localized and the $[Ca^{2+}]_i$ changes were measured under different experimental conditions. Fura-2 fluorescent measurements were performed with a DeltaScan illumination system (Photon Technology International, South Brunswick, NJ), and data acquisition and analysis were performed using FELIX 1.1 software (Photon Technology International).

Data analysis

Data are expressed as mean ± SE. SigmaPlot software (Jandel Scientific, San Rafael, CA) was used for estimates of reversal potential values.

RESULTS

SP-induced inward current in TRP3C(+)/NK2R(+) cells

HEK293 cells transiently transfected with NK2R-EGFP were located by their green fluorescence signal (Fig. 1A). Wild-type cells with green fluorescence, transfected with NK2R-EGFP but not with TRP3C, did not show a measurable response (>20 pA) respond to bath applied SP (1 µM, Fig. 1B, $n = 12$). Nonfluorescent HEK293 cells stably transfected with TRP3C were also studied as another control. None of these responded to bath-applied 1 µM SP (Fig. 1C, $n = 6$). Nonetheless, all six cells showed an inward current ($3.8 ± 1.32$ pA/pF, $n = 6$) to bath applied 10 or 100 µM carbachol, suggesting the presence of functional TRP3C (Hurst et al. 1998). Cells containing both NK2R and TRP3C, selected by the presence of EGFP among the cells stably transfected with TRP3C, generated an inward current on SP application (Fig. 1D). The mean peak SP-evoked current density was $11.3 ± 3.48$ pA/pF ($n = 24$), ranging from 0.4 pA/pF to 85.7 pA/pF (Table 1). The time course of response was relatively slow, indicated by long time to reach the peak of the response ($195 ± 120.0$ s, $n = 20$). Recording at room temperature might explain this slow time course.

Pharmacology of SP-induced responses

We used an NK2R agonist and antagonist to test the specificity of SP responses recorded in NK2R transfected HEK293 cells. Application of NKA (1 µM), a NK2R agonist, induced an inward current with a mean current density of $11.0 ± 3.40$ pA/pF ($n = 8$), which is similar to SP-induced responses. NKA-induced currents tended to reach the peak of the response faster ($116 ± 25.1$, $n = 8$) than SP-induced currents ($195 ± 120.0$, $n = 20$) but this difference was not statistically significant. High affinity of NKA for the NK2R might explain this

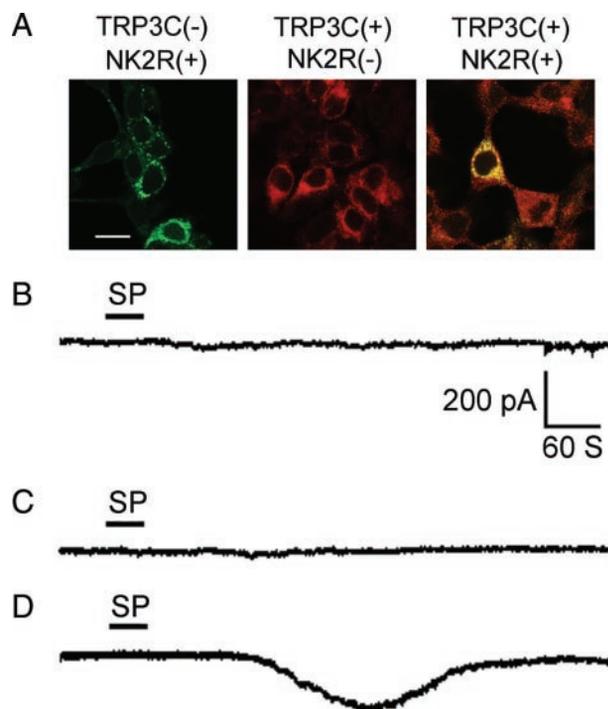


FIG. 1. Substance P (SP) induced currents in HEK 293 cells transfected with TRP3 channels (TRP3C) and/or NK2 receptors (NK2R). *A*: representative HEK293 cells for each group of transfections. Green and red fluorescence represents NK2R and TRP3C, respectively. Yellow is the overlap of both colors, indicating the presence of both NK2R and TRP3C. Scale bar denotes 20 μm . *B*: application of SP (1 μM) for 40 s did not evoke currents in HEK293 cells expressing NK2R only. *C*: HEK293 cells expressing TRP3C only also did not show any current change on SP application. *D*: in HEK293 cells expressing both TRP3C and NK2R, SP application induced a slow inward current, lasting several minutes. There were variable delays between SP application and current onset. In this particular cell, there was a delay of approximately 1.5 min. Current traces were recorded at a holding potential of -80 mV. Scale bar applies to all 3 traces. Horizontal bars depict time of SP application.

trend of faster time course. Pretreatment of HEK293 cells with a NK2R antagonist, SR48968 (1 μM), completely blocked (Fig. 2A) the 1- μM SP- or 1- μM NKA-induced responses ($n = 7$). To evaluate whether these ionic currents were through TRP3C, we applied Gd^{3+} or SKF96365, known TRPC antagonist (Inoue et al. 2001). The decay of SP-induced inward current tended to be accelerated when Gd^{3+} (1 μM) was applied just after inward current approached peak (Fig. 2B). The recovery time for the SP response, measured as the time required to decay 50% from peak amplitude, was 54 ± 7.1 s (range, 44–68 s; $n = 3$) in the presence of Gd^{3+} compared with 104 ± 23.4 s (range, 64–207 s; $n = 6$) without Gd^{3+} .

TABLE 1. Characteristics of substance P responses in HEK293 cells expressing TRP3 channels and NK2 receptors

Measurement	Mean \pm SE
Peak current density (pA/pF)	11.3 ± 3.48 (24)
Time to peak (s)	195 ± 120.0 (20)
Reversal potential (mV)	6.8 ± 7.66 (6)

Values are mean \pm SE with number of responses in parentheses. Peak current density was calculated by dividing current size at the peak of the response by cell membrane capacitance. Time to peak was measured as the duration between the initiation and the peak of the SP response. Reversal potential value was obtained by determining the membrane potential where control and SP ramp currents intersect (see Fig. 3).

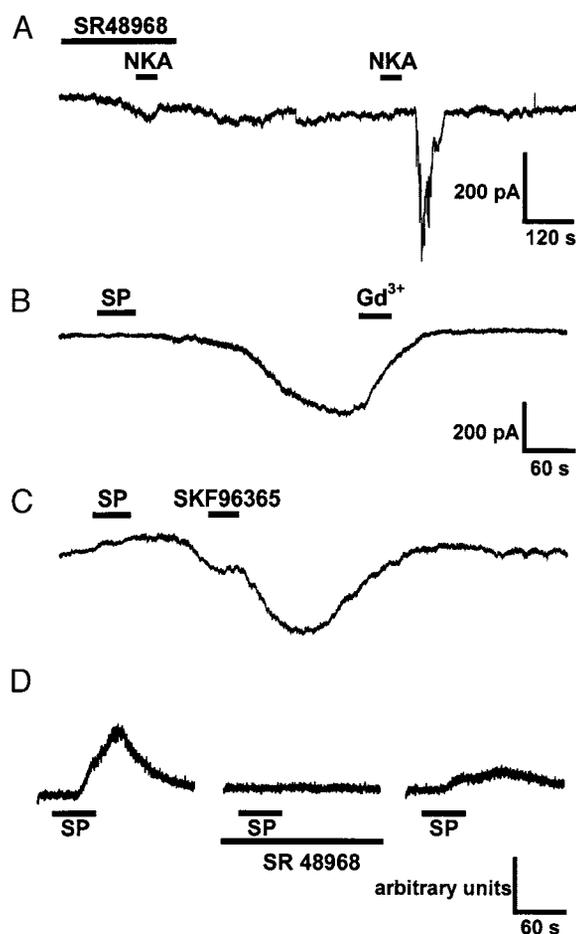


FIG. 2. Pharmacology of SP responses in HEK293 cells transfected with TRP3C and NK2R. *A*: application of the NK2R agonist NKA (1 μM , 40 s) in the presence of the selective NK2R antagonist SR48968 (1 μM , 3 min) did not induce any current change. After an 8-min washout of SR48968, NKA produced a large inward current. *B*: a nonspecific cation channel blocker, Gd^{3+} (1 μM , 30 s), was applied at the peak of the SP response. Gd^{3+} facilitated the return of current to baseline. *C*: a blocker of TRP3C, SKF96365 (30 μM , 30 s) decreased the development of an inward current produced by SP application. After switching to normal Locke solution, ionic current further progressed to reach peak. Currents were recorded at a membrane potential of -80 mV. Lower scale bar applies to current traces in *B* and *C*. *D*: intracellular Ca^{2+} was increased on SP (1 μM) application. This Ca^{2+} rise was completely blocked by SR48968 (1 μM). After 5-min washout, SP-induced Ca^{2+} response partially recovered.

Although there was clear effect of Gd^{3+} on the decay kinetics of the SP response, there was no statistical difference between the two groups ($P = 0.199$). When SKF96365 (30 μM) was applied during the inward current induced by SP, it reduced the amplitude of the current or halted further increase of inward current (Fig. 2C, $n = 6$). It inhibited $58 \pm 6.9\%$ of peak current induced by SP. On washout of SKF96365, SP-evoked inward currents resumed, reached a peak, and then returned to baseline. The absence of measurable SP-evoked membrane currents in HEK293 cells that lack NK2R or TRP3C along with pharmacological results indicates that activation of NK2R in HEK293 cells links to the activation of an inward current through TRP3C.

SP-induced intracellular calcium rise

In HEK293 cells with NK2R and TRP3C, intracellular Ca^{2+} was increased when SP was bath-applied ($n = 36$, Fig. 2D).

Intracellular Ca^{2+} rose slowly with a monophasic response, which was different from the carbachol-induced response (an initial transient component was followed by a sustained component, data not shown). This response was completely blocked by $1 \mu\text{M}$ SR48968, a NK2R antagonist, with partial recovery on removal of the antagonist ($n = 3$, Fig. 2D).

Membrane conductance change and reversal potential

To evaluate the ionic bases of the SP-induced inward currents, we monitored conductance changes and measured reversal potential (E_{rev}) values using voltage ramp commands. The membrane potential was depolarized from -80 to $+60$ mV at a rate of 0.5 mV/ms and each ramp was elicited every 3 s. During the SP-induced response, the membrane conductance was increased, depicted by the increase in the amplitude of inward and outward transient ramp currents (Fig. 3A) and an increased slope of ramp current (Fig. 3B). E_{rev} value was estimated by plotting an I - V relation for control and for the SP response. The membrane potential where ramp current traces

intersect one another provides an estimate of the E_{rev} value for the SP-induced responses (Fig. 3B). In all cells studies ($n = 6$), SP increased the membrane conductance, with the mean E_{rev} value 6.8 ± 7.66 mV (Table 1), suggesting that SP evokes a nonspecific cationic current.

DISCUSSION

In the cell bodies of either primary vagal afferents (nodose ganglion neurons, NGNs, Moore et al. 1999; Weinreich et al. 1997) or those in primary somatosensory afferents (dorsal root ganglion neurons, DRGNs, Inoue et al. 1995), SP evokes a membrane depolarization that is associated with an increase in membrane conductance. The reversal potential value (E_{rev}) for SP in NGNs, measured by sharp microelectrode technique, was around -27 mV; the E_{rev} for SP in DRGNs were near 10 mV, measured by whole cell patch-clamp technique. These observations suggest that SP may activate nonselective cation channels in primary sensory nerve cells; the nature of these channels, however, remains unknown.

The current experiments with HEK293 cells transfected with NK2Rs and TRPCs were performed to explore the hypothesis that TRPCs may be the final effectors of activated SP receptors in primary afferent neurons. There are several reasons to consider that activation of TRPCs can underlie SP-induced cationic currents. TRPCs are nonselective cation channels (Clapham et al. 2001) that are activated by components of the inositol phospholipid hydrolysis system, the same system activated by tachykinin receptor signaling (Otsuka and Yoshioka 1993). In NGNs, the SP-evoked inward current is dependent on an elevation of intracellular Ca^{2+} (Moore et al. 1999), suggesting an involvement of an inositol phospholipid system. Finally, TRPC mRNA and protein are expressed in nodose ganglia (Garcia and Schilling 1997; D. Kunze, personal communication).

Our major finding was that SP evoked an inward current and an elevated intracellular Ca^{2+} in HEK293 cells transfected with NK2R and with TRP3 channels (TRP3C). The reversal potential value of approximately 7 mV of this current indicated that it was a nonspecific cation conductance. The SP-evoked current was due to activation of NK2Rs because the NK2R agonist NKA mimicked the SP response and either SP or NKA responses were blocked by a specific NK2R receptor antagonist, SR48968. The SP-evoked currents were partially blocked by Gd^{3+} and SKF96365, drugs that block ionic conductance through TRP channels. Finally, SP-evoked inward currents were absent in HEK293 cells lacking either NK2R or TRP3C. Together, these observations indicate that tachykinin receptors activation can cause the opening of TRPC. These results also provide a rationale for considering that nonselective cation channels coupled with tachykinin receptors in mammalian primary sensory neurons may belong to the TRPC family of cation channels.

The reversal potential value E_{rev} for the SP response in NGNs was around -27 mV, while those in DRGNs and transfected HEK293 cells had E_{rev} values of 10 and 7 mV, respectively. The disparity in E_{rev} values for SP-evoked currents recorded in NGNs versus DRGNs and HEK-293 cells may reflect different recording conditions (sharp microelectrode vs. whole cell patch), existence of different heterotetram-

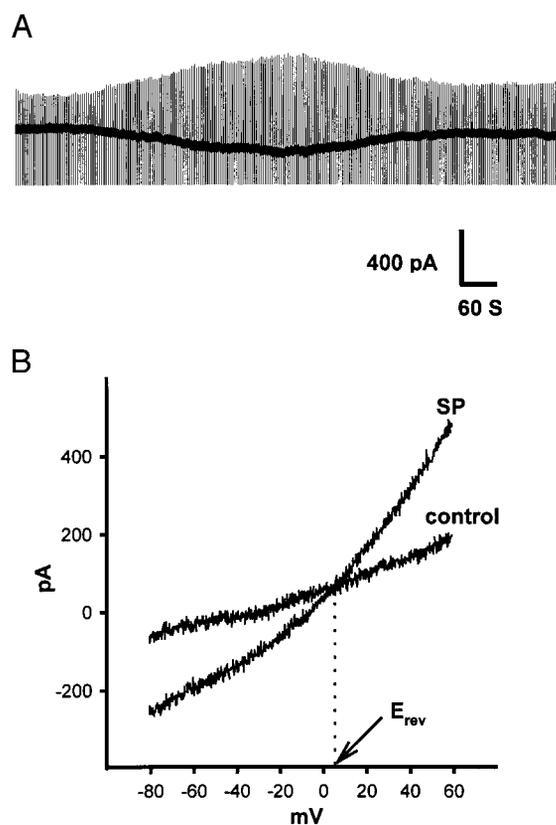


FIG. 3. Effect of SP on membrane conductance of HEK293 cells transfected with TRP3C and NK2R. *A*: SP application ($1 \mu\text{M}$, 40 s) induced an inward current that was associated with increased membrane conductance. Vertical bars represent repetitive (0.33 Hz) ramp current evoked by ramp voltage-clamp commands (-80 to $+60$ mV, 0.5 mV/ms). During the SP response, the amplitude of the ramp currents was increased indicating a conductance increase. *B*: I - V relationships were plotted for ramp currents recorded at baseline (control) and at the peak of the SP response. The slope of the ramp current was steeper on application of SP, suggesting SP increased the membrane conductance. The reversal potential (E_{rev}) was obtained by determining the membrane potential value where SP did not change membrane current (e.g., where both traces intersect). For this cell, E_{rev} was $+6$ mV. Peak current density was calculated by dividing current size at the peak of the response by cell membrane capacitance.

ers of four single subunits of TRP channels or the presence of different TRP channel accessory proteins.

SP induced slow inward currents with increased intracellular Ca^{2+} only in the cells with both NK2Rs and TRPC expression. The specificity of these responses was further supported by pharmacological studies. An NK2R antagonist could completely block these responses and an NK2R agonist could mimic them. NKA, which has higher affinity than SP to NK2Rs, induced faster inward currents but their mean current density and time to peak were not significantly different from those measured for SP-induced currents. Gd^{3+} is known as a nonspecific TRPC blocker with an IC_{50} of 1.9 μM for TRPC6 expressed in HEK293 cells (Inoue et al. 2001). To block SP-induced currents, 1 μM Gd^{3+} was applied when SP-induced current reached the peak. Gadolinium could not block the current completely but was able to accelerate the recovery of current back to baseline. Another nonspecific TRPC blocker, SKF96365, was applied after the initiation of SP-induced current. In each case that SKF96365 was applied, it partially inhibited the development of inward current. However, after washout of SKF96365, SP-induced currents resumed their normal trajectory (Fig. 2C). These results demonstrate that, while TRPC blockade reduces SP-induced current, it does not interfere with SP-induced activation of TRPC. The E_{rev} values for SP-induced currents ranged from -2 to $+18$ mV. Similar E_{rev} values have been reported for SP-induced currents in other systems (Inoue et al. 1995; Moore et al. 1999) and in TRPC currents activated by other ligands (Li et al. 1999). In addition, preliminary data revealed that SKF96365 (30 μM) can also inhibit SP-induced currents in NGNs ($65 \pm 12.0\%$ inhibition of the peak current, $n = 5$). This result supports our hypothesis that SP-induced currents in NGNs may be carried by TRPC. Further work using antisense to various TRPC may help clarify the role of TRPC in tachykinin signaling in NGNs and other primary sensory afferent neurons.

In conclusion, SP was able to evoke inward currents and intracellular Ca^{2+} rises in HEK293 cells where NK2R and TRPC3 were expressed together. This finding suggests that TRPC might be the nonspecific cation channel that is responsible for the effect of tachykinin receptor activation.

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DISCLOSURES

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