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cDNA cloning of bovine substance-K receptor through oocyte expression system

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The neuropeptide receptors which are present in very small quantities in the cell and are embedded tightly in the plasma membrane have not been well characterized. Mammals contain three distinct tachykinin neuropeptides, substance P, substance K and neuromedin K, and it has been suggested that there are multiple tachykinin receptors[1,2]. By electrophysiological measurement, we have previously shown that *Xenopus* oocytes injected with brain and stomach mRNAs faithfully express mammalian substance-P and substance-K receptors, respectively[3]. Here we report the isolation of the cDNA clone for bovine substance-K receptor (SKR) by extending this method to develop a new cloning strategy. We constructed a stomach cDNA library with a cloning vector that allowed *in vitro* synthesis of mRNAs and then identified a particular cDNA clone by testing for receptor expression following injection of the mRNAs synthesized *in vitro* into the oocyte system. Because oocytes injected with exogenous mRNAs can express numerous receptors and channels, our new strategy will be applicable in the general molecular cloning of these proteins. The result provides the first indication that the neuropeptide receptor has sequence similarity with rhodopsin-type receptors (the G-protein-coupled receptor family) and thus possesses multiple membrane-spanning domains.

The principle of the new cloning strategy is illustrated in Fig. 1. A mixture of complementary DNAs was first synthesized from bovine stomach messenger RNA by using a specific poly(dT) primer. This primer contains multiple restriction sites next to the poly(dT) tail so that when an appropriate enzyme is chosen, cleavage of one of these sites terminates the subsequent *in vitro* transcription of cDNAs cloned in λ immediately after the poly(A) tail but leaves the receptor mRNA sequence intact. After synthesis of single- and double-stranded cDNAs, the resultant cDNA mixture was integrated into the *Eco*RI site located downstream from the SP6 promoter in the λ vector DNA. We chose λ as a cloning vector, because it is capable of incorporating a large DNA insert, increasing the proportion of clonal cDNAs encoding whole mRNA sequences. Appropriate numbers of λcDNA clones were plated after *in vitro* packaging[4] and the clonal λcDNA mixture was extracted and cleaved with an appropriate restriction enzyme. The resultant λcDNA mixture was transcribed *in vitro* by specific SP6 RNA polymerase in the presence of the capping nucleotide[5]. The mRNA mixture synthesized was injected into oocytes which were then tested for the expression of the receptor by measuring electrophysiological response to application of substance K. Following our experimental design, a mixture of cDNA clones comprising the receptor cDNA sequence would be identified by detecting the electrophysiological response of oocytes injected with the mRNA to application of substance K. The receptor cDNA clone could subsequently be purified by stepwise fractionations of the response-evoking cDNA mixture on the basis of the *in vitro* mRNA synthesis and electrophysiological measurement.

We began by examining SKR expression for the λcDNA mixture comprising $\sim 3 \times 10^5$ cDNA clones. A clear response to substance K was observed for oocytes injected with the mRNA derived from the cDNA mixture. We therefore attempted to purify the cDNA clone for SKR by following the ability to evoke a response through stepwise fractionations of the cDNA clone mixture. Testing fractions containing ~50,000, ~20,000, ~2,000 and 83 λcDNA clones, we finally obtained a single clone (λSKR56) that was capable of inducing electrophysiological response to substance K. When an *Eco*RI fragment containing the whole cDNA sequence of λSKR56 was inserted immediately downstream from either the SP6 promoter or the T7 promoter in the plasmid DNA (pSKR56S or pSKR56T), the mRNA synthesized *in vitro* by SP6 RNA polymerase (pSKR56S) or T7 RNA polymerase (pSKR56T) again induced an electrophysiological response to substance K in the oocyte expression system (see Fig. 4). Thus, we concluded that this cDNA sequence is sufficient for the induction of an electrophysiological response to substance K. The SKR cDNA was sequenced by the chain termination method[6].

Figure 2 shows the 2,458-nucleotide sequence of the cloned cDNA and the amino-acid sequence deduced for SKR, which was assigned from the longest open-reading frame of the cDNA sequence. This polypeptide consists of 384 amino-acid residues (relative molecular weight (M_r) = 43,066). The hydropathicity profile[7] of the deduced amino-acid sequence and a comparison of its sequence with those of other proteins[8] revealed seven hydrophobic segments and significant sequence similarity shared with rhodopsin-type receptors[9–15]. Figure 3 shows the alignment of the amino-acid sequences for SKR and rhodopsin-type receptors (β_2-adrenergic receptor, βAR; muscarinic acetylcholine receptors, M_1AChR and M_2AChR; and opsin). The degree of sequence identity is 24, 24, 22 and 21% for SKR-βAR, SKR-M_1AChR, SKR-M_2AChR and SKR-opsin comparisons, respectively.

Rhodopsin-type receptors have a structure consisting of seven hydrophobic membrane-spanning domains with an extracellular amino-terminus and a cytoplasmic carboxy-terminus[9,16,17]. A similar transmembrane model for SKR, with seven membrane-spanning segments, can be suggested from its primary structure (Fig. 3). The seven putative transmembrane α-helices of SKR consist of a continuous stretch of 20–24 uncharged amino-acid residues, except that segments II, V and VI contain Asp 79, His 198 and His 267, respectively. The residue corresponding to Asp 79 is conserved for all rhodopsin-type receptors. Several cysteine residues (corresponding to residues 106, 181, 262, 309 and 324) are also conserved in all or most of the receptors. In contrast to the putative transmembrane domains, the amino- and carboxy-terminal regions and the third cytoplasmic loop (loop V–VI) differ in length and in the amino-acid sequence between SKR and the rhodopsin-type receptors. However, these regions of SKR also show a pattern similar to the rhodopsin-type receptors[17], including potential *N*-glycosylation sites[18] (Asn 11 and Asn 19) in the amino-terminal region with no signal sequence[19], and the presence of many Ser and Thr residues in the carboxy-terminal region as possible phosphorylation sites[17]. Thus, the close similarities between SKR and rhodopsin-type receptors, in both their amino-acid sequences also general structural features, indicate that SKR belongs to the family of rhodopsin-type receptors, and suggest that SKR is associated with GTP-binding proteins[17] that effect the second-messenger system of SKR function[3,20].

Because the single cDNA clone encodes a functional SKR, we investigated the specificity of SKR with the oocyte expression system. The three mammalian tachykinins all showed electro-

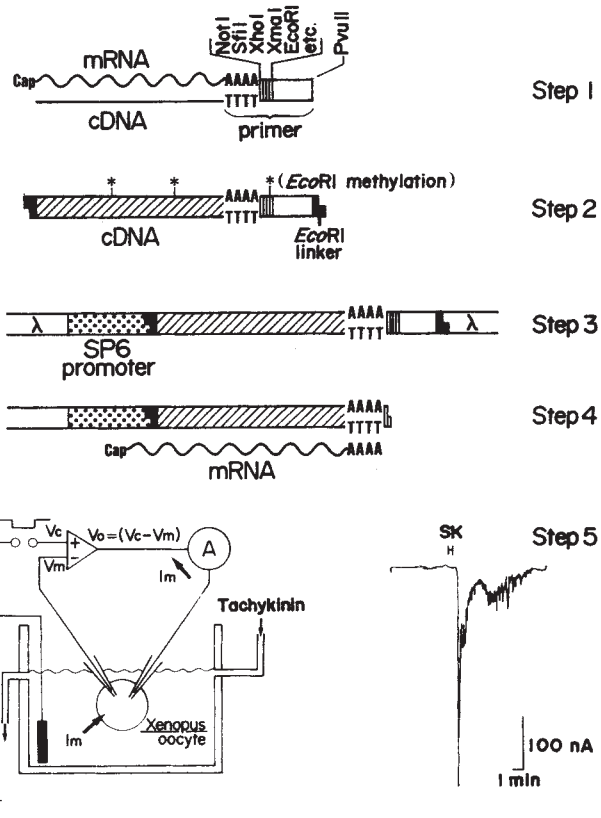

Fig. 1 Strategy for isolating a cDNA clone for bovine SKR. Clone λSKR56 containing the bovine SKR cDNA was isolated as follows. Step 1: To make the primer DNA, the chemically synthesized oligonucleotides containing rare *Not*I and *Sfi*I sites were inserted into the *Sal*I site in the polylinker of pUC19 DNA (ref. 23).

5′-TCGAGCGGCCGCCATGGCCTCGAG-3′
3′-CGCCGGCGGTACCGGAGCTCAGCT-5′

The modified pUC19 DNA (pUC19NS) obtained by transformation was cleaved at the *Pst*I site. About 70 thymidine residues were added to the 3′ end of the *Pst*I site[4] and the DNA fragment containing the poly(dT) tail was isolated after *Pvu*II cleavage. This cDNA primer contained the following restriction sites next to the poly(dT) tail: *Not*I-*Nco*I-*Sfi*I-*Xho*I-*Sal*I-*Xba*I-*Bam*HI-*Xma*I-*Kpn*I-*Sac*I-*Eco*RI. Bovine stomach poly(A) RNA was subjected to sucrose density gradient centrifugation[3], and a fraction giving potent SKR expression in the oocyte expression system was used for cDNA synthesis. Step 2: The single-stranded cDNAs were converted to double-stranded DNAs[24], and *Eco*RI sites were added to both ends of the cDNA sequences through the *Eco*RI linker ligation method[24]. Step 3: The λ vector DNA containing SP6 promoter was constructed as follows. The *Nae*I site of pGEM2 plasmid DNA (Promega Biotec) was converted to the *Bam*HI site by *Bam*HI linker ligation, and the *Bam*HI fragment containing the SP6 promoter was then inserted into the *Bam*HI site of λgt10 after being converted from the single *Eco*RI site of the original λgt10 (ref. 25). The λDNA vector (λgt10SP6) thus constructed contained a single *Eco*RI site immediately downstream of the SP6 promoter, and the cDNA mixture described in step 2 was inserted in this *Eco*RI site. A bovine stomach λcDNA library was then constructed by *in vitro* packaging[4]. Step 4: DNA was extracted from a mixture of $\sim 3 \times 10^5$ clones derived from the stomach λcDNA library and digested with *Not*I. Digested DNA was then transcribed *in vitro* by SP6 RNA polymerase in the presence of the cap analogue GpppG as described[5]. Step 5: After digestion of template DNA with DNaseI, the mRNAs synthesized *in vitro* were injected into oocytes, and their electrophysiological response to application of substance K was measured by the conventional two-micropipette voltage clamp method[3]. The purification of the cDNA clone for SKR was then carried out by repeating step 4 and step 5 after stepwise fractionations of the response-evoking cDNA mixtures. Finally, a single clone (λSKR56) was obtained. A typical substance K response evoked by the mRNA derived from λSKR56 is also shown: substance K-responsive current (downward deflection indicates inward current) was recorded under voltage clamp at −60 mV. V_c, command potential; V_m, membrane potential; V_o, output of differential amplifier; I_m, current through cell membrane; A, current monitor.

Fig. 2 The cDNA sequence for bovine SKR and its deduced amino-acid sequence. Nucleotide residues are numbered in the 5′ to 3′ direction, beginning with the first residue of the initiation methionine codon of a long open-reading frame of the cDNA sequence, and the nucleotides on the 5′ side of residue 1 are indicated by negative numbers. The predicted amino-acid sequence of SKR is shown above the nucleotide sequence. The cDNA sequence for SKR was determined by the chain termination method after subcloning the cDNA insert of λSKR56 into M13mp18 and M13mp19 (ref. 23).

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                                                                                CTGATCCCTAATGTCTCCTGAACCACATTAGGAGGGACCTGGGTTTGAGTTTATCACCAACTTACTACATGACCTGG  -361

GCTGGTCCCCACTTGGAATGTTTTTCCATTTCTGCAGTGAAGATGCAGGCTGATGATTTAGAAGGATGCCTCTTACTCTGAGACCACGTCTTGGATTCTAGATATCCACGTGACGTTGGA  -241

GAAGGCTGGCCAGGACATGGGAATCTGAAGTGCCTAGGGCTCTGGACCCGGCTGTCTGCTCAGCCTAACTCATCTCTGTTTGAGACTTGGGAGTGACCCCCCAGCTCCCTGGACGCGCCT  -121

GGCTCGGCACCTACTCCTCCTGCTCCCCGCACTCAGAGAGGATTTGTGTGCTCCCACAGGGCACCAGGGCCTGGAGGGAGCCAAGGAGCCTTACCTCTTGTGCCAGACTCAGAGGCGGCC   -1

  1                  10                  20                  30                  40
MetGlyAlaCysValValMetThrAspIleAsnIleSerSerGlyLeuAspSerAsnAlaThrGlyIleThrAlaPheSerMetProGlyTrpGlnLeuAlaLeuTrpThrAlaAlaTyr
ATGGGGGGCTGTGTCGTGATGACCGATATCAACATCTCGTCTGGCCTTGACAGCAATGCCACGGGCATCACAGCCTTCTCCATGCCCGGCTGGCAGCTGGCACTGTGGACCGCAGCCTAC  120

 41                  50                  60                  70                  80
LeuAlaLeuValLeuValAlaValMetGlyAsnAlaThrValIleTrpIleIleLeuAlaHisGlnArgMetArgThrValThrAsnTyrPheIleValAsnLeuAlaLeuAlaAspLeu
CTGGCCCTGGTGCTGGTGGCTGTGATGGGCAATGCCACAGTCATCTGGATCATTCTGGCCCATCAGAGGATGCGCACGGTGACCAACTACTTCATCGTCAACCTGGCCCTGGCCGACCTC  240

 81                  90                  100                 110                 120
CysMetAlaAlaPheAsnAlaAlaPheAsnPheValTyrAlaSerHisAsnIleTrpTyrPheGlyArgAlaPheCysTyrPheGlnAsnLeuPheProIleThrAlaMetPheValSer
TGCATGGCTGCCTTCAACGCTGCCTTCAACTTCGTCTACGCCAGCCACAACATCTGGTACTTTGGCCGTGCCTTCTGCTACTTCCAAAACCTCTTCCCCATCACAGCCATGTTCGTCAGT  360

121                 130                 140                 150                 160
IleTyrSerMetThrAlaIleAlaAlaAspArgTyrMetAlaIleValHisProPheGlnProArgLeuSerAlaProGlyThrArgAlaValIleAlaGlyIleTrpLeuValAlaLeu
ATCTACTCCATGACTGCCATTGCTGCTGACAGGTACATGGCCATCGTCCACCCCTTCCAGCCCCGGCTCTCAGCTCCTGGCACCAGAGCGGTCATTGCTGGCATCTGGCTGGTGGCCCTG  480

161                 170                 180                 190                 200
AlaLeuAlaProProGlnCysPheTyrSerThrIleThrThrAspGluGlyAlaThrLysCysValValAlaTrpProGluAspSerGlyGlyLysMetLeuLeuLeuTyrHisLeuIle
GCCCTCGCCTTCCCCCAATGCTTCTATTCCACCATCACCACGGATGAGGGTGCCACCAAGTGCGTGGTGGCCTGGCCTGAAGACAGCGGTGGCAAGATGCTCCTTTTGTACCACCTCATA  600

201                 210                 220                 230                 240
ValIleAlaLeuIleTyrPheLeuProLeuValValMetPheValAlaTyrSerValIleGlyLeuThrLeuTrpArgArgSerValProGlyHisGlnAlaHisGlyAlaAsnLeuArg
GTGATCGCCCTCATTTACTTCCTGCCTCTCGTGGTGATGTTCGTCGCCTACAGTGTCATCGGACTCACGCTCTGGAGACGCTCTGTCCCGGGGCACCAGGCTCATGGTGCCAACTTGCGC  720

241                 250                 260                 270                 280
HisLeuGlnAlaLysLysLysPheValLysThrMetValLeuValValValThrPheAlaIleCysTrpLeuProTyrHisLeuTyrPheIleLeuGlyThrPheGlnGluAspIleTyr
CACCTGCAGGCCAAGAAGAAGTTCGTGAAGACCATGGTGCTGGTGGTGGTGACGTTTGCCATCTGCTGGCTGCCCTACCACCTCTACTTCATCCTGGGCACCTTCCAGGAGGACATCTAC  840

281                 290                 300                 310                 320
CysHisLysPheIleGlnGlnValTyrLeuAlaLeuPheTrpLeuAlaMetSerSerThrMetTyrAsnProIleIleTyrCysCysLeuAsnHisArgPheArgSerGlyPheArgLeu
TGCCACAAGTTCATCCAGCAGGTCTACCTGGCGCTCTTCTGGCTGGCCATGAGCTCCATCATTTACTGCTGCCTCAACCACAGGTTTCGCTCTGGATTCCGGCTT  960

321                 330                 340                 350                 360
AlaPheArgCysCysProTrpValThrProThrGluGluAspLysMetGluLeuThrTyrThrProSerLeuSerThrArgValAsnArgCysHisThrLysGlullePheMetMetSer
GCTTTCCGTTGCTGTCCATGGGTCACACCAACCGAGGAGGACAAGATGGAGCTGACCTACACTCCATCCCTCTCCACGAGGGTCAACAGGTGTCACACTAAAGAGATTTTTTTCATGTCT  1080

361                 370                 380  384
GlyAspValAlaProSerGluAlaValAsnGlyGlnAlaGluSerProGlnAlaGlyValSerThrGluPro
GGGGATGTGGCCCCCTCTGAGGCTGTCAACGGGCAGGCTGAAAGCCCCCAGGCTGGAGTGTCTACTGAACCCTAAAGCCTAGTGCCTGGCTGCAGGGAGAGGCGTCCGCACCCTTGAGAA  1200

GTAGCCAGTCGAAAGGACCACCTCTGAAAGTTTGGCCCGTGCCTCACCCTTCAGTACATCAATGGAAAGATGAGATGGGGCCAGGAACTCTGCAGTGGGTCCTGCCTCTCCCAGCCAGCC  1320

CCTCAGACAGTGCTTCAGGGGATCCAATAATTCAAAGAAGACAGGGTGAGCTAACACTTATCTAATCCAGGAGTTGCAAGGTCGAATGCCTTCCTGGGGCATACAACATAGAGTAAATTG  1440

AACCCGGTATGAAAGAAAAATTGTAGATATATTGATAAAAGATAACTGACTTCAGTGTCGGGCCAATAGGGAATGGTGGGGACTGGCAAACTGGAGGGCATAGGAATCCAAAGGGGAGAG  1560

TAGCTGATCAGCTCTGGGCAAATGCTGCCATGTGACTGATCCTCTACATCTTTCAAGAGAAGCCAGAAATCCAAATTTGTGAAACTTCCAGAATTGTTAAATTTGGCTCACATTGTTATA  1680

AATGTTGTGCGAACCAAACAAAACACACCAGTGGGCAGTACTCAACCATGACACTGATTTATTATCAGGATAAAGACTCAAGGAAAGAAGCTGCTTAATTCTGCAAAGCCCCTAACCTGT  1800

GAGCCAATCCAGGGCTTCTCTGCTTTAGCATCTAAGAACCCAGGTCCACCCCAGGGGAGATGGTGGTTAAGGGTCAAAAGAAGCCTCAGCTGGGTGGTCTCAAGACAATGACCTTCACAT  1920

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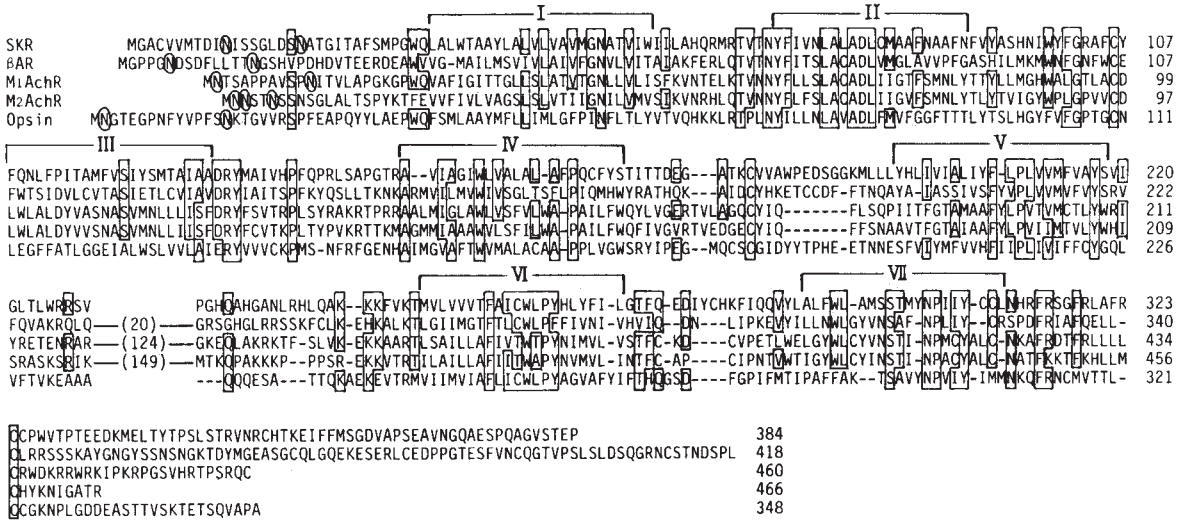
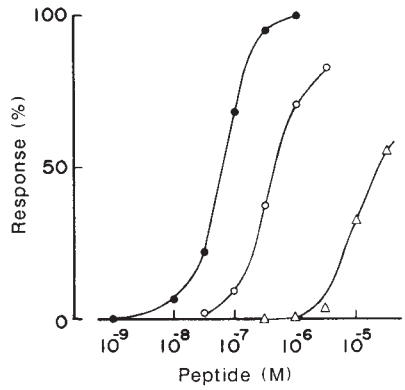


Fig. 3 Alignment of the amino-acid sequences of bovine SKR (top), hamster β_2-adrenergic receptor (βAR, second row), porcine cerebral and cardiac muscarinic acetylcholine receptors (M_1AChR, third row and M_2AChR, fourth row) and bovine opsin (bottom row). (Data for the four latter sequences from refs 11–15). The amino-acid residues enclosed by solid lines represent residues which are identical in at least two of the four reference sequences when compared with the SKR sequence; the non-homologous sequences in loop V–VI are indicated by the sum of residues present in each segment given in parentheses. The degree of sequence identity described in the text was calculated by counting a continuous stretch of gaps as one substitution regardless of its length. Positions of the putative transmembrane segments I–VII of SKR are indicated; the termini of each segment are tentatively assigned on the basis of hydropathicity profile, amino-acid sequence and comparison with rhodopsin-type receptors[13,17]. Circled residues, Asn residues that are potential *N*-glycosylation sites in amino-terminal regions.

Fig. 4 Dose–response curves of substance K (●), neuromedin K (○) and substance P (△) in inducing electrophysiological response of oocytes injected with mRNA derived from cloned SKR cDNA. **Methods.** For experimental convenience, the cDNA insert of λSKR56 was transferred to the plasmid pGEM1 DNA (Promega Biotec); the *Eco*RI fragment containing the whole cDNA sequence together with its following poly(dT-dA) tail and the multiple restriction sites was isolated from λSKR56 and inserted into the *Eco*RI site immediately downstream from the SP6 promoter or the T7 promoter of pGEM1 DNA (pSKR56S and pSKR56T). After digestion of the plasmid DNA with *Not*I, DNA was transcribed by SP6 RNA polymerase (pSKR56S) or T7 RNA polymerase (pSKR56T) in the presence of the capping nucleotide[5]. The mRNA was injected into oocytes (mRNA concentration, 2 ng μl^{-1}; average volume injected per oocyte, ~40 nl). The injected oocytes were incubated at 20 °C for 2–3 days. Electrophysiological measurements were performed at 20–22 °C in Ringer's solution. Responses to tachykinins were recorded under voltage clamp at 0 mV. Each point represents the mean of at least five data.



physiological induction, as indicated in Fig. 4, while no response was induced by the application of the peptides tested that were not tachykinins. The half-maximal concentrations of substance K, neuromedin K and substance P for electrophysiological induction were determined to be 6.0×10^{-8} M, 3.4×10^{-7} M and 8.7×10^{-6} M, respectively. Thus, SKR is the receptor most selective for substance K, but it also has the ability to cross-react with neuromedin K and substance P.

The oocyte system can be used to produce numerous foreign channels as well as their associated neurotransmitter receptors, depending on the source of mRNA injected[21,22]. These include the receptors for amines, amino acids and neuropeptides[3,22]. Furthermore, this system is extremely sensitive; a single SKR clone in the mixture of $\sim3\times10^5$ cDNA clones still produced a clear response to substance K. No convenient biochemical tools for obtaining the purified protein have been available to date for most channels and receptors, but our method will be widely applicable as a direct approach to isolating cDNA clones for these proteins.

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