The mechanism by which angiotensin directly affects ovulation remains to be determined. In preliminary experiments Ang II added to ovarian follicles that were being cultured in vitro increased the level in the culture medium of prostaglandin F2 α and plasminogen activator, both of which have been associated with LH action and specifically with ovulation (*10*).

Thus, this phylogenetically ancient regulatory system, which had previously been considered important only in maintaining fluid and electrolyte balance and vascular tone, also plays a direct role in reproduction (*11, 12*). Furthermore, our findings raise the possibility that among the several groups of compounds active on the renin-angiotensin system there may be some with potential as novel forms of contraception or as profertility drugs.

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In Situ Transcription: Specific Synthesis of Complementary DNA in Fixed Tissue Sections

LAURENCE H. TECOTT, JACK D. BARCHAS, JAMES H. EBERWINE*

A technique, in situ transcription, is described, in which reverse transcription of mRNAs is achieved within fixed tissue sections. An oligonucleotide complementary to proopiomelanocortin (POMC) mRNA was used as a primer for the specific synthesis of radiolabeled POMC cDNA in fixed sections of rat pituitary, thus permitting the rapid anatomical localization of POMC mRNA by autoradiography. Intermediate lobe signal intensities were sensitive to dopaminergic drugs, demonstrating that the method can be used for studies of mRNA regulation. The transcripts may also be eluted from tissue sections for a variety of uses, including the identification and cloning of autoradiographically localized cDNAs from small amounts of tissue.

THE SYNTHESIS OF cDNA BY THE enzyme reverse transcriptase is typically performed in a solution reaction with extracted RNA serving as a template. We report here a method whereby mRNA can serve as a template for this enzyme in situ, within a fixed tissue section. The in situ transcription (IST) procedure (Fig. 1) is initiated by in situ hybridization of an oligonucleotide complementary to a specific mRNA, providing a primer that is required for the polymerase activity of reverse transcriptase (*1*). This priming step allows one to select the individual mRNA to be transcribed. The enzyme then extends the primer with the mRNA acting as a template. During the polymerase reaction, radiolabeled deoxynucleotides may be incorporated into transcripts, resulting in high specific activity cDNA.

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The IST method was developed by examining POMC gene expression in the rat pituitary. The POMC gene gives rise to a family of biologically active peptides including adrenocorticotropin (ACTH), β-endorphin, and α-melanocyte–stimulating hormones (α-MSH) (*2*). An oligonucleotide 36 nucleotides (nt) in length, complementary to the sequence encoding amino acids 100 to 111 of rat POMC, was used as the primer (*3*). After in situ hybridization to fresh frozen paraformaldehyde-fixed sections of rat pituitary (11 μm thick), reverse transcription was performed in the presence of ^{35}S-labeled deoxycytidine with the other three deoxynucleotides unlabeled. A strong signal was observed in the intermediate lobe (Fig. 2, a and c), consistent with the known localization of POMC mRNA (*4–8*). When the primer was omitted from the hybridization mix, this signal was not observed (Fig. 2b). Omission or heat inactivation of the reverse transcriptase also eliminated the signal. As a negative control, reverse transcription was performed after a hybridization step with a 36-nt probe complementary to tyrosine hydroxylase mRNA (*9*), which has not been detected in the rat pituitary (*10*). Accordingly, no signal above background was pro-

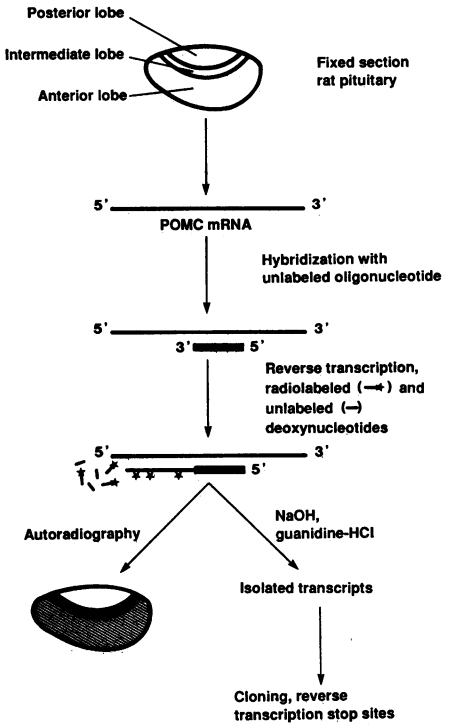

Fig. 1. Schematic outline of the in situ transcription (IST) procedure for the detection of POMC mRNA. An unlabeled oligonucleotide is hybridized to POMC mRNA, followed by reverse transcription within the tissue section in the presence of radiolabeled deoxynucleotides. Autoradiography is then performed for the localization of radiolabeled POMC cDNA. After autoradiography, the transcripts may be denatured and eluted by NaOH or guanidine-HCl treatments, and the transcripts may be either cloned or separated by electrophoresis for subsequent analysis.

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duced (Fig. 2d). As a positive control, reverse transcription was performed after hybridization to polyadenylated [poly(A^+)] mRNAs with a polythymidine oligomer 36 nt in length. Intense signals in both the anterior and intermediate lobes were observed (Fig. 2e), consistent with the abundance and expected distribution of poly(A^+) mRNA.

To further confirm the specificity of the POMC IST signal, we performed in situ transcription in pituitary sections from animals that had been treated with the dopamine antagonist haloperidol or the dopamine agonist bromocriptine. The intensities of the intermediate lobe signals varied in a manner consistent with the known dopaminergic regulation of POMC mRNA (*4–6, 11–15*). The neuroleptic haloperidol, which elevates POMC mRNA levels (*4, 11, 13*), increased the intermediate lobe IST signal (Fig. 2g) relative to control (Fig. 2f). Conversely, the dopamine agonist bromocriptine greatly reduced the signal (Fig. 2h), consistent with the effect of this drug on levels of POMC mRNA in the intermediate lobe (*4, 13*).

A striking feature of the intermediate lobe POMC IST signal was its intensity. Exposure times of 10 min were required for film autoradiographs with ^{35}S-labeled transcripts, in contrast with the 3-hour exposures required for in situ hybridization with 3′ end ^{35}S-labeled oligonucleotide probes. When the IST reaction was performed in the presence of [^{3}H]deoxycytidine, an emulsion autoradiographic exposure of only 15 hours was sufficient for the cellular localization of silver grains in the intermediate lobe (Fig. 3). The increase in signal intensity relative to standard protocols (*16*) likely resulted from the incorporation into IST transcripts of many radiolabeled deoxynucleotides (approximately 25% of incorporated deoxynucleotides) per mRNA molecule.

A primer-independent background signal was observed that did not require a hybridization step before the enzyme reaction but did require reverse transcriptase (Fig. 2b). This suggested that endogenous sites occurred in the tissue section, serving as primer-template complexes for the initiation of reverse transcriptase activity. To confirm this, reverse transcription with radiolabeled deoxynucleotides was performed in sections that had not received a prior oligonucleotide hybridization step. After denaturation, the eluted material was run on a polyacrylamide-urea sequencing gel. A smear with few discrete bands was evident after film autoradiography (Fig. 4a, lane 1), indicating that high molecular weight transcripts had been synthesized. This endogenous priming could be due to DNA-DNA, DNA-RNA, or RNA-RNA duplexes, all of which can serve as initiation sites for reverse transcriptase activity (*17*).

To characterize the transcripts produced in the POMC IST reaction, we eluted radiolabeled POMC oligonucleotide-primed IST transcripts from pituitary sections and separated them on a denaturing gel. Complementary DNA transcripts had been produced and formed a complex banding pattern (Fig. 4a, lane 3). The IST transcripts produced after hybridization with the heterologous tyrosine hydroxylase oligonucleotide formed a smear of radioactivity (Fig. 4a, lane 2) similar to that of the unprimed sample (Fig. 4a, lane 1) (*18*). To determine whether the bands represented extensions of the oligonucleotide primer, we hybridized with a 5′ end ^{32}P-labeled POMC oligonucleotide to the pituitary section, followed by reverse transcription in the presence of high concentrations of unlabeled deoxynucleotides. The resulting transcripts produced a similar autoradiographic pattern of bands (Fig. 4b), thus demonstrating that they resulted from extension of the oligonucleotide primer.

To determine whether the POMC oligo-

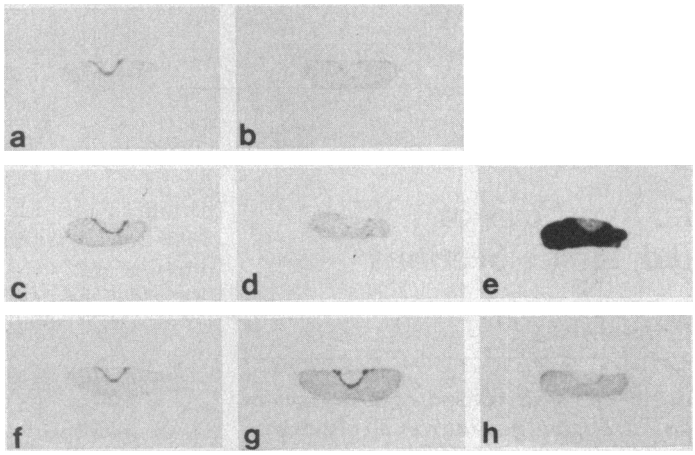

Fig. 2. In situ transcription of POMC mRNA in fixed pituitary sections. Cryostat sections (11 μm thick) were prepared from fresh frozen pituitaries. Sections were fixed for 5 min in 3% neutral buffered paraformaldehyde. In situ hybridization was performed for 12 to 16 hours at room temperature in a mixture consisting of 4× standard saline citrate (SSC) and 50% formamide, to which was added unlabeled oligonucleotide (1 ng/25 μl). Sections were washed, with two changes, in 2× SSC for 30 min, at room temperature, followed by washes in 0.5× SSC, at 40°C for 2 hours, with one change. Reverse transcription was performed at 37°C for 1 hour in a reaction mixture consisting of 50 m*M* tris-HCl (*p*H 8.3); 6 m*M* $MgCl_2$; 40 m*M* KCl; 7.5 m*M* dithiothreitol; 250 μ*M* deoxyadenosine triphosphate (dATP); thymidine triphosphate (TTP); and deoxyguanosine triphosphate (dGTP); ^{35}S-labeled α-dCTP (300 μCi/ml, 1000 Ci/mmol, Amersham); 0.12 unit of ribonuclease inhibitor (RNasin) per microliter (Bethesda Research Laboratories); and 600 units of avian myoblastosis virus reverse transcriptase per milliliter (Seikagaku). Sections were then washed, with two changes of 2× SSC for 30 min each, at room temperature, followed by washes in 0.5× SSC, at 40°C for 6 hours with two changes. Sections were then dehydrated and apposed to Kodak XAR x-ray film for 10 min before development. Drug treatments: rats received daily intraperitoneal injections of bromocriptine (3 mg per kilogram of body weight) (Sandoz), haloperidol (McNeil), or vehicle for 4 days and were killed 24 hours after the last injection. (**a** and **c**) Autoradiograms of IST with POMC oligonucleotide primer demonstrating intermediate lobe signal (posterior lobe above and anterior lobe below the intermediate lobe). (**b**) Oligonucleotide omitted from the hybridization mix. (**d**) Tyrosine hydroxylase oligonucleotide primer. (**e**) Polythymidine primer. (**f**) POMC IST in vehicle-, (**g**) haloperidol-, and (**h**) bromocriptine-treated animals.

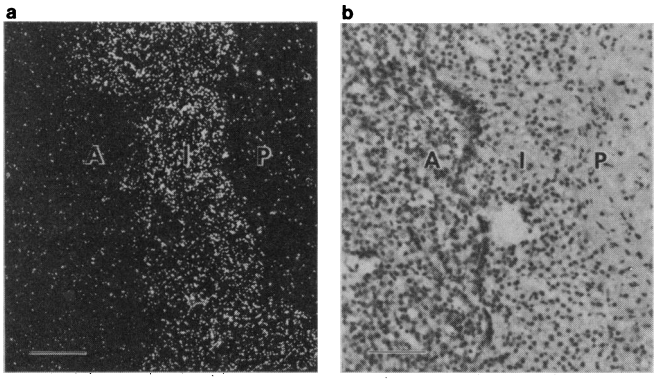

Fig. 3. Localization of POMC IST transcripts in rat pituitary by emulsion autoradiography. IST procedure as described in Fig. 2, except that 60 μCi of α-[^{3}H]dCTP per milliliter (50 Ci/mmol, Amersham) replaced ^{35}S-labeled α-dCTP in the reverse transcriptase reaction. The section was dipped in Kodak NTB2 nuclear track emulsion, diluted 1:1 with water. After a 15-hour exposure, the autoradiogram was developed in Kodak D19 for 2 min at 17°C and fixed in Kodak fixer for 5 min at 17°C. Counterstaining was done with hematoxylin and eosin. (**a**) Dark-field photomicrograph revealing a dense band of silver grains in the intermediate lobe (I), as compared with posterior (P) or anterior (A) lobes. (**b**) Bright-field photomicrograph of same field. Silver grains not visible at this magnification with bright-field optics. Scale bar, 100 μm.

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nucleotide-primed IST transcripts were cDNA copies of POMC mRNA, we hybridized the denatured transcripts to single-stranded POMC cDNA that had been immobilized on nitrocellulose filters (*19*). After high-stringency washes, the hybridized transcripts were eluted from the filter and separated by electrophoresis on a polacrylamide-urea gel. The banding pattern before hybridization (Fig. 4c, lane 6) was similar to that observed for transcripts that had hybridized to POMC cDNA (Fig. 4c, lane 7), whereas the nonhybridizing transcripts did not produce bands on electrophoresis (Fig. 4c, lane 8). This identified the bands as sequences complementary to POMC cDNA.

The specific cDNAs produced by the IST reaction may also be denatured and eluted from tissue sections for cloning. It was possible to clone POMC cDNA from three 11-μm-thick pituitary sections by using this method (*20*). Since these sections contained roughly 4.5 ng of poly(A^+) mRNA, cloning of specific cDNAs by IST may be achieved with a smaller amount of tissue than is required by other methods [in which more than 1 μg of poly(A^+) mRNA is usually required for the generation of high complexity libraries]. This feature of the technique may prove useful, for example, when cloning human cDNAs from limited quantities of pre- or postmortem tissues. Another advantage of cloning by the IST method is that the precise anatomical localization of the mRNAs to be cloned may be determined by autoradiography. The specificity of transcription provided by the priming step is particularly important in tissues that have a complex population of mRNAs, such as the brain (*21*). The method potentially obviates the need to make cDNA libraries of high complexity for specific cDNA isolation. In preliminary studies, we have combined the IST technique with the polymerase chain reaction (*22*) to make highly complex cDNA libraries from four pituitary sections (*23*).

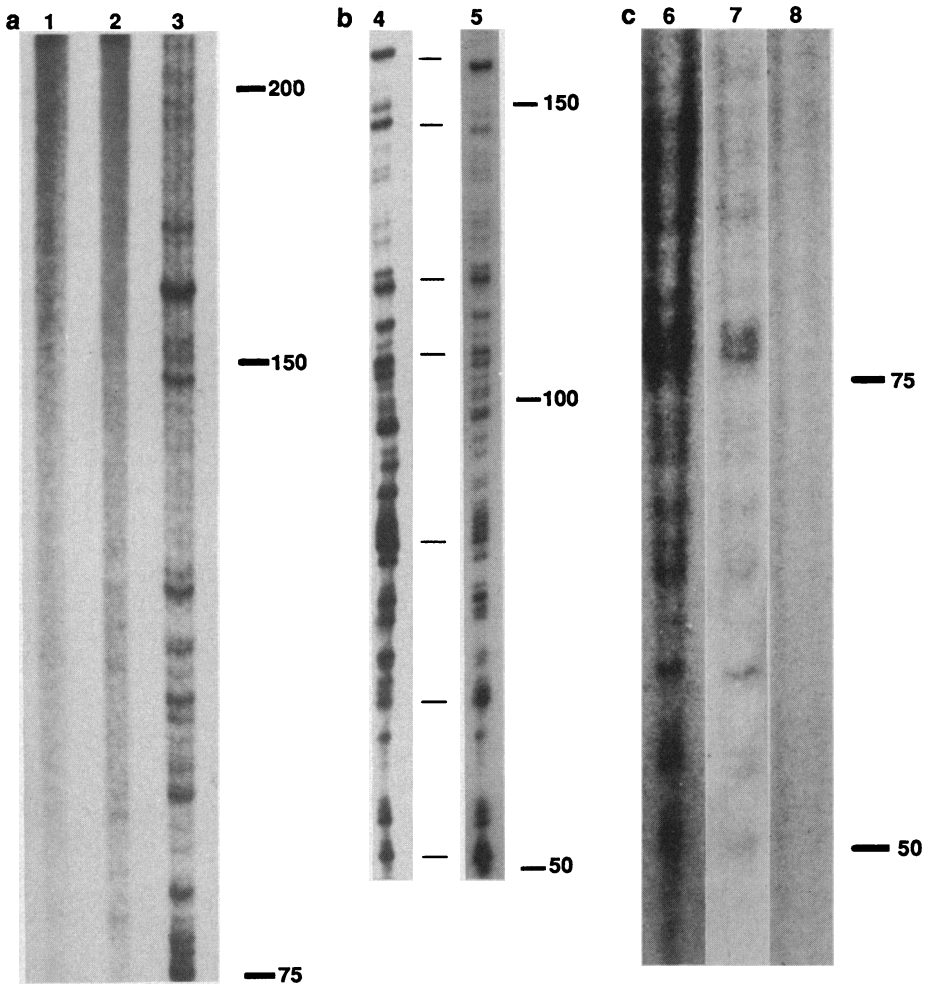

Fig. 4. Electrophoresis of IST transcripts. All POMC IST reactions used oligonucleotide primers as described in text. The IST procedure was performed as described in Fig. 2, except that 300 μCi of α-[^{32}P]dCTP per milliliter (410 Ci/mmol, Amersham) replaced ^{35}S-labeled α-dCTP in the reverse transcriptase reaction. For gel electrophoresis, transcripts were denatured by incubating sections in (50 μl per section) 4*M* guanidine-HCl and 1*M* β-mercaptoethanol for 1 hour at 23°C. This was followed by protein extraction with 0.5 volume of phenol and 0.5 volume of chloroform, with subsequent ethanol precipitation (10 mg glycogen per milliliter, 2 volumes ethanol, 30 min in dry ice) before electrophoresis. Size markers represent lengths in nucleotides. (**a**) Five percent polyacrylamide and 7*M* urea gel, 3000 cpm loaded per lane. Transcripts resulting from priming with no oligonucleotide (lane 1), heterologous 36-nt oligonucleotide (lane 2), or POMC oligonucleotide (lane 3). (**b**) Five percent polyacrylamide and 7*M* urea gel. Comparison of (lane 4) POMC-primed IST cDNA transcribed in the presence of α-[^{32}P]dCTP (3000 cpm loaded) and (lane 5) transcripts primed by a 5′ end, ^{32}P-labeled POMC oligonucleotide (specific activity, 2×10^8 cpm/μg), followed by reverse transcription in the presence of 250 μ*M* unlabeled deoxynucleotides (900 cpm loaded). (**c**) Six percent polyacrylamide and 7*M* urea gel. Comparison of (lane 6) POMC-primed IST transcripts after elution from tissue, (lane 7) POMC-primed IST transcripts after elution from tissue and hybridization to POMC cDNA that had been immobilized on nitrocellulose filters (*25*). After hybridization in 30% formamide, 5× SSC, 100 μg salmon sperm DNA per milliliter, and 5× Denhardt's, the filters were washed in 2× SSC and 0.1% SDS for 30 min at 37°C followed by two washes in 0.2× SSC and 0.1% SDS for 30 min at 42°C. The bound IST transcripts were eluted by incubating the filter in a solution containing 0.1% SDS, 0.5 m*M* EDTA at 65°C for 30 min, followed by ethanol precipitation; (lane 8) IST transcripts that did not hybridize to the POMC cDNA–bound filter.

The origin of banding patterns observed after gel electrophoresis of POMC oligonucleotide-primed IST transcripts is of interest. The bands were unlikely to have been the result of degradation of POMC mRNA, which would be expected to produce a smear upon autoradiography. It is also unlikely that the bands resulted from limiting deoxycytidine triphosphate (dCTP) concentrations (*23*), as indicated by the appearance of a similar pattern of bands from transcripts that were primed by a 5′ end ^{32}P-labeled oligonucleotide and extended in the presence of high concentrations of all four deoxynucleotides. The IST banding patterns may have resulted from the termination of reverse transcription at discrete sites along the POMC mRNA molecule. Such patterns may be unique to the sequence of the transcribed template and may be useful for the differentiation of viral subtypes and the alternate splice products of a single gene.

Because reverse transcription was performed in tissue sections where cellular constituents may interact with mRNA, it is possible that the polymerase reaction was hindered by such associations. The analysis of the products of reverse transcription may therefore provide information concerning the secondary structure of the template and the association of ribosomes or proteins with the mRNA.

In summary, the IST technique makes it possible to synthesize cDNA copies of specific mRNAs within fixed tissue sections. The specificity of the oligonucleotide-

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primed synthesis of POMC cDNA in fixed pituitary sections was demonstrated by: (i) intermediate lobe localization of the autoradiographic signal, (ii) primer dependence and specificity of the signal, (iii) patterns of signal intensity in drug-treated animals consistent with the known regulation of POMC mRNA, and (iv) the hybridization to POMC cDNA of IST transcripts, which formed a discrete pattern of bands upon electrophoresis. The ability to generate cDNA in tissue sections, without the need for RNA extraction procedures, permits the rapid anatomical localization of mRNA. The sensitivity of the generated autoradiographic POMC IST signals to dopamine agonists and antagonists demonstrates that the method may be used in studies of the regulation of mRNA levels. In addition, cDNA may be obtained from small amounts of tissue, potentially permitting the cloning of specific cDNAs from tissues for which cDNA libraries do not currently exist.

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Intracellular Injection of cAMP Induces a Long-Term Reduction of Neuronal K^+ Currents

KENNETH P. SCHOLZ* AND JOHN H. BYRNE

Intracellular signals that trigger long-term (24-hour) changes in membrane currents in identified neurons of *Aplysia* have been examined in order to understand the cellular mechanisms underlying long-term sensitization. Adenosine 3′,5′-monophosphate (cAMP) was directly injected into individual sensory neurons to mimic the effects of sensitization training at the single cell level. Potassium currents of these cells were reduced 24 hours after injection of cAMP; these currents were similar to those reduced 24 hours after behavioral sensitization. These results suggest that cAMP is part of the intracellular signal that induces long-term sensitization in *Aplysia*.

THE LARGE AND IDENTIFIABLE NEURONS of the mollusk *Aplysia* have made it a useful model to study cellular changes in neurons that accompany alterations of behavior (*1*). For example, long-term sensitization of certain reflex responses can last for several days (*2, 3*). These behavioral changes are associated with changes in (i) morphology of sensory neuron synaptic contacts (*4*), (ii) amplitude of evoked excitatory postsynaptic potentials (EPSPs) from sensory neurons to motor neurons (*5, 6*), (iii) sensory neuron excitability (*7, 8*), and (iv) membrane currents of the sensory neurons (*3*). In addition, these changes require protein synthesis (*6, 9*). The intracellular signals that lead to the induction of long-term sensitization are unknown. Because cAMP is elevated in the sensory neurons during the application of the stimuli that lead to long-term sensitization (*10*), we have assessed the role of cAMP

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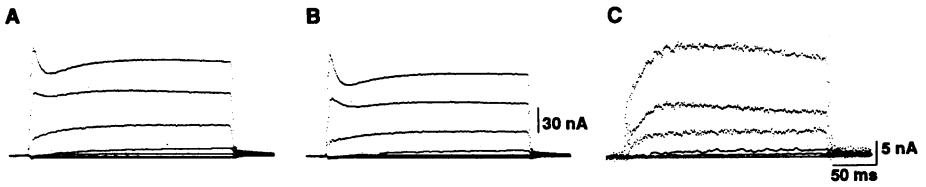

Fig. 1. Membrane currents in response to voltage-clamp pulses from a holding potential of −50 mV. Voltage-clamp pulses were elicited to membrane potentials between −80 and +25 mV in steps of 15 mV. (**A**) Overall average of current response families representing all cells injected with 5′-AMP 24 hours before voltage clamping. (**B**) Overall average of current response families representing all cells injected with cAMP 24 hours before voltage clamping. (**C**) The averaged cAMP difference families [(A) − (B)] from all ten clusters. The current response family in (C), therefore, represents the net outward currents that are reduced as a consequence of previous injection of cAMP (I_{cAMP}). Note different vertical calibration in (C).

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