water, the problem is directly approached through temperature dependence studies. For reactions in organic solvent, there must be further investigations into the magnitude of the effect.

In the foreseeable future time-resolved photoacoustic calorimetry will be extended onto the 1-ns time scale, given the recent developments in piezoelectric transducers and transient-recording devices. The technique should find wide ranging application to problems in chemistry and biochemistry that include solid-state reactions and dynamics of proteins in membranes.

REFERENCES AND NOTES

1. A. G. Bell, *Philos. Mag.* **11**, 510 (1881).
2. W. R. Harshbarger and M. B. Robin, *Acc. Chem. Res.* **6**, 329 (1973).
3. C. K. N. Patel and A. C. Tam, *Rev. Mod. Phys.* **53**, 517 (1981).
4. A. C. Tam, *ibid.* **58**, 381 (1986).
5. M. W. Sigrist, *J. Appl. Phys.* **60**, R83 (1986).
6. D. R. Ort and W. W. Parsons, *Biophys. J.* **25**, 355 (1979).
7. T. A. Moore, D. Benin, R. Tom, *J. Am. Chem. Soc.* **104**, 7356 (1982).
8. L. J. Rothberg, J. D. Simon, M. Bernstein, K. S. Peters, *ibid.* **105**, 3464 (1983).
9. J. E. Rudzki, J. L. Goodman, K. S. Peters, *ibid.* **107**, 7849 (1985).
10. S. E. Braslavsky, in *Primary Photo-Processes in Biology and Medicine*, R. V. Bensasson, G. Jori, E. J. Land, Eds. (Plenum, New York, 1985), pp. 147–158.
11. S. J. Komorowski and E. M. Eyring, *J. Appl. Phys.* **62**, 3066 (1987).
12. K. Heihoff and S. E. Braslavsky, *Chem. Phys. Lett.* **131**, 183 (1986).
13. J. A. Westrick, J. L. Goodman, K. S. Peters, *Biochemistry* **26**, 8313 (1987).
14. K. S. Peters, in *Kinetics and Spectroscopy of Carbenes and Biradicals*, M. Platz, Ed. (Plenum, New York, in press).
15. J. L. Goodman, K. S. Peters, H. Misawa, R. A. Caldwell, *J. Am. Chem. Soc.* **108**, 6803 (1986).
16. J. L. Goodman, K. S. Peters, V. Vaida, *Organometallics* **5**, 815 (1986).
17. G. K. Yang, K. S. Peters, V. Vaida, *Chem. Phys. Lett.* **125**, 566 (1986).
18. S. P. Nolan, *et al.*, *J. Am. Chem. Soc.* **109**, 3143 (1987).
19. K. Heihoff, S. E. Braslavsky, K. Schaffner, *Biochemistry* **26**, 1422 (1987).
20. S. E. Braslavsky, *Pure Appl. Chem.* **56**, 1153 (1984).
21. M. Jabben, K. Heihoff, S. E. Braslavsky, K. Schaffner, *Photochem. Photobiol.* **40**, 361 (1984).
22. M. Jabben *et al.*, *ibid.* **43**, 127 (1986).
23. W. P. Leung, K. C. Cho, S. K. Chau, C. L. Choy, *Chem. Phys. Lett.* **141**, 220 (1987).
24. J. C. Kendrew *et al.*, *Nature* **185**, 422 (1960).
25. T. Takano, *J. Mol. Biol.* **110**, 569 (1977).
26. D. A. Case and M. Karplus, *ibid.* **132**, 343 (1979).
27. J. L. Martin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 173 (1983).
28. E. R. Henry, J. H. Sommer, J. Hofrichter, W. Eaton, *J. Mol. Biol.* **166**, 443 (1983).
29. S. A. Rudolph, S. O. Boyle, D. F. Dresden, S. J. Gill, *Biochemistry* **11**, 1098 (1972).
30. D. Ringe, G. A. Petsko, D. E. Kerr, P. R. Ortiz de Montellano, *ibid.* **23**, 2 (1984).
31. J. L. Goodman and K. S. Peters, *J. Am. Chem. Soc.* **108**, 1700 (1986).
32. J. M. Kanabus-Kaminska, J. A. Hawari, D. Griller, C. Chatgiliarloglu, *ibid.* **109**, 5267 (1987).
33. T. J. Burkey, M. Majewski, D. Griller, *ibid.* **108**, 2218 (1986).
34. J. A. Lavilla and J. L. Goodman, *Chem. Phys. Lett.* **141**, 149 (1987).
35. N. A. Garcia, G. Rossbroich, S. E. Braslavsky, H. Durr, C. Dorweiler, *J. Photochem.* **31**, 297 (1985).
36. R. Bonneau, *ibid.* **10**, 439 (1979).
37. R. A. Caldwell and C. V. Cao, *J. Am. Chem. Soc.* **104**, 6174 (1982).
38. J. A. Welch, K. S. Peters, V. Vaida, *J. Phys. Chem.* **86**, 1941 (1982).
39. K. E. Lewis, D. M. Golden, G. P. Smith, *J. Am. Chem. Soc.* **106**, 3905 (1984).

40. This work has been supported by grants from the National Science Foundation and the National Institute of Health.

Heterologous Expression of Excitability Proteins: Route to More Specific Drugs?

HENRY A. LESTER

Many clinically important drugs act on the intrinsic membrane proteins (ion channels, receptors, and ion pumps) that control cell excitability. A major goal of pharmacology has been to develop drugs that are more specific for a particular subtype of excitability molecule. DNA cloning has revealed that many excitability proteins are encoded by multigene families and that the diversity of previously recognized pharmacological subtypes is matched, and probably surpassed, by the diversity of messenger RNAs that encode excitability molecules. In general, the diverse subtypes retain their properties when the excitability proteins are expressed in foreign cells such as oocytes and mammalian cell lines. Such heterologous expression may therefore become a tool for testing drugs against specific subtypes. In a systematic research program to exploit this possibility, major considerations include alternative processing of messenger RNA for excitability proteins, coupling to second-messenger systems, and expression of enough protein to provide material for structural studies.

The author is in the Divison of Biology, California Institute of Technology, Pasadena, CA 91125.

MANY DRUGS IN THE MODERN PHARMACOPOEIA ACT ON ion channels, receptors, and ion pumps in biological membranes. These molecules belong to several families of intrinsic membrane proteins that I term collectively "excitability proteins." Two facts help to explain the medical importance of drug effects on excitability proteins. (i) Because excitability proteins function at the cell surface, they are accessible to drugs present in the extracellular fluid. (ii) Because these proteins usually control cellular events that operate on a time scale of milliseconds to minutes, their activation or inhibition can often be rapidly monitored by the physician and readily appreciated by the patient.

The available drugs, despite their utility, have many side effects. This article concerns a particular type of side effect that seems to arise because many excitability proteins are members of multigene families: there are undesired interactions with homologous excitability proteins in tissues or cells that are not the drug's intended target.

I cite here a few examples of such side effects. These examples are chosen because their protein targets span the known classes of excitability molecules.

1) The β-adrenergic receptor blockers, such as propranolol, are effective antihypertensive agents because of their actions on the cardiovascular system. However, they may cause fatigue, depression, and other effects related to actions on β-adrenergic receptors in the central nervous system.

2) The dihydropyridine (DHP) Ca^{2+} channel blockers, such as nifedipine, are useful in treating angina pectoris. However, they can also cause orthostatic hypotension by blocking Ca^{2+}-mediated contraction of peripheral arterial smooth muscle.

3) Benzodiazepines are useful in treating status epilepticus, presumably because they potentiate the action of γ-aminobutyric acid (GABA) at its receptors, thereby decreasing muscle spasms. Yet actions on GABA receptors in other cells depress brainstem respiratory control centers, which can lead to respiratory arrest, especially in children.

4) Antipsychotic drugs such as phenothiazines (for example, chlorpromazine), thioxanthenes, butyrophenones, and diphenylbutylpiperidines are thought to exert their action by blocking dopamine receptors in the mesolimbic system. However, these drugs produce neurological side effects, including parkinsonism, through blockade of dopamine receptors in the basal ganglia.

5) The cardiac glycoside digoxin, which blocks the Na^+,K^+–adenosine triphosphatase (ATPase), is still an important therapeutic tool for congestive heart failure. Patients with an excess of circulating thyroid hormone have reduced sensitivity to cardiac glycosides, possibly because they express a glycoside-resistant form of the ATPase (*1, 2*). The higher required dosage for these patients could be toxic to other organs in which the ATPase retains its normal sensitivity.

A new approach for a systematic program to develop more specific drugs has simultaneously occurred to several investigators. This approach is based on the expression of excitability molecules from DNA clones in cells that both readily support such expression and can readily be studied with the full range of modern physiological and pharmacological techniques. To develop a useful new pharmaceutical is a long process that draws on many disciplines. In this article I emphasize only the specificity of drug action, without concern for other factors such as pharmacokinetics and toxicity of metabolites.

Scientific Basis

Two recent accomplishments have made possible this new paradigm of drug development.

Cloning. Primary amino acid sequences have been deduced by cDNA cloning for several excitability molecules, and clear patterns have emerged (Table 1). (i) Several receptors fall into a class of proteins that contain seven transmembrane helices and interact with guanine nucleotide binding (G) proteins. (ii) Another group comprises ligand-gated receptor-channel complexes; there are several distinct but homologous subunits, each containing about four transmembrane helices and an extracellular loop that seems to bind the agonist. (iii) A group of voltage-gated channel proteins contain a peptide with one to four homologous "motifs," each containing about six transmembrane regions. One of the putative membrane-spanning regions contains four to six repeats of the Arg-X-X or Lys-X-X sequence, where X is a nonpolar amino acid residue. This region may constitute the voltage sensor. (iv) A family of ion-stimulated ATPases serves to establish concentration gradients across membranes; members have six to ten transmembrane helices. Each of these groups may gain many more members over the next few years, and each group contains clinically important excitability proteins.

It is likely that cloning will reveal other families of excitability molecules. For instance, no channel gated by an intracellular ligand has yet been cloned or sequenced. Two examples would be (i) the guanosine 3′,5′-monophosphate (cGMP)–activated channels of vertebrate photoreceptors and olfactory cells; and (ii) the ATP-suppressed K^+ channel (*3*). Another group might be represented by the amiloride-sensitive, voltage-insensitive Na^+ channel of transporting epithelia.

Expression. The second set of accomplishments concerns progress in DNA- or RNA-mediated functional expression of these clones in the membrane of foreign cells (Table 2). This procedure, termed heterologous expression, is a useful form of reconstitution. The favorite expression system has been injection of in vitro–synthesized mRNA into *Xenopus* oocytes; such expression often serves as decisive proof of a successful cloning project. Indeed, in several recent cases, oocyte expression has served as the primary assay for isolation of a cDNA clone (*4–6*).

Like oocytes, mammalian cells can be induced to express cloned excitability molecules transiently; monkey COS cells are a particularly well characterized system (*7*). Like oocyte injection, however, such transient expression has the major drawback that the nucleic acids must be prepared and applied to the cells for each separate experiment.

Therefore, stably transfected mammalian cell lines are a desirable expression system. Table 2 lists several successful transfection experiments with such systems involving members of the seven-helix family. A notable achievement concerns the stable transfection of all four subunits for the *Torpedo* nicotinic acetylcholine (ACh) receptor. The expressed receptors display many hallmarks of normal receptors,

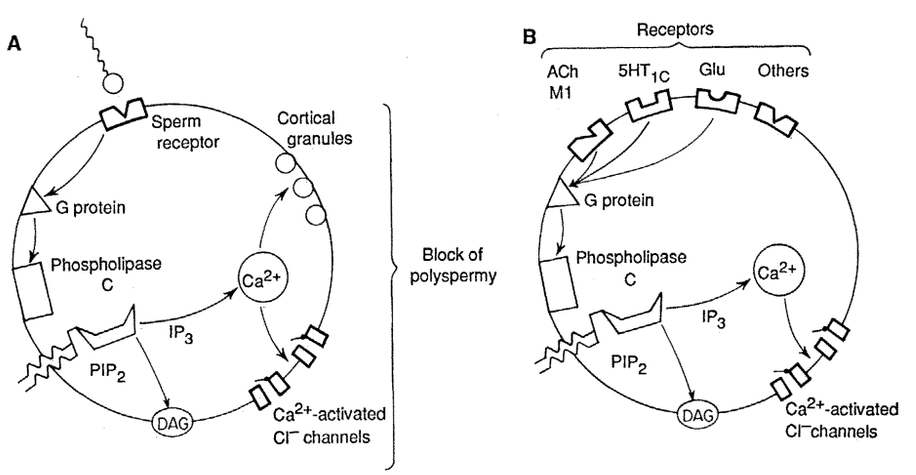


Fig. 1. Simplified view of the relation between expressed receptors and the endogenous phosphoinositide pathway in *Xenopus* oocytes and mature eggs (*52*). (**A**) In the mature egg, a putative sperm receptor couples to a G protein, which in turn activates phospholipase C. The role of diacylglycerol (DAG) is unclear; but the inositol trisphosphate (IP_3) releases Ca^{2+} from intracellular stores (*100*). The elevated Ca^{2+} concentration activates Cl^- channels that depolarize the egg, preventing further sperm from fusing to the plasma membrane (*101*). (More slowly, the elevated Ca^{2+} concentration causes the cortical granules to fuse with the plasma membrane, raising the fertilization envelope, which forms a mechanical barrier to sperm entry.) (**B**) In the immature oocyte, several seven-helix receptors are expressed by injection of the appropriate RNA. These receptors couple to the endogenous G protein and eventually activate the same Cl^- channels, providing a sensitive assay for expressed receptors (*102*). (The cortical granules do not fuse in immature oocytes.) Abbreviations: Glu, glutamate; and PIP_2, phosphatidylinositol-4,5-bisphosphate.

including α-bungarotoxin and dimethyl *d*-tubocurarine binding properties, sedimentation properties, single-channel conductance and lifetime, and desensitization (*8*). Yeast represents another potentially useful expression system (*9*). Several other strategies theoretically represent more desirable expression systems but remain untested with excitability molecules (*10*).

The extent of functional reconstitution varies with the protein expressed and with the expression system (Table 2). In some cases, only agonist or antagonist binding has been reported. In other cases, there is detectable coupling via G proteins to enzymes of intracellular messenger pathways such as adenylate cyclase. For the particular case of coupling to phospholipase C, the receptor signal in oocytes is amplified by an endogenous pathway that constitutes part of the mature egg's response to sperm (Fig. 1). The resulting sensitivity allowed the detection of a single full-length cDNA clone for a serotonin 1C ($5HT_{1C}$) receptor in a library containing 10^6 other clones (*6*).

Functional reconstitution of membrane channels is best assessed with electrophysiological measurements. For both the receptor-channel complex family and the voltage-gated channel family, macroscopic and single-channel voltage-clamp currents have provided the most meaningful assay of functional reconstitution (Table 2).

Diversity. An additional point emerges from recent experiments with cloning, with sequence-specific antibodies, and with gel chromatographic analysis of excitability proteins. The diversity of previously recognized pharmacological and electrophysiological subtypes is matched, and probably surpassed, by the diversity of genes that encode receptors and channels (Tables 1 and 3). The binding of specific radioligands showed that receptors and channels can be distinguished by many subtypes. For example, muscarinic ACh receptors contain two (M1 and M2) or three subtypes, which can be distinguished by their affinity for pirenzepine and for AF-DX 116 (*11*). Also, channels have often been subclassified in terms of kinetics and single-channel properties. For example, voltage-dependent Ca^{2+} channels have been classified with several epithets, such as L, T, and N (*12*). There are also variations in the sensitivity to cardiac glycosides and antigenicity between subforms of Na^+,K^+-ATPase (*1, 13*). It may be asked whether pharmacological and electrophysiological distinctions such as these result from differences in posttranslational processing or in primary amino acid sequence. In many cases, as summarized in Tables 1 and 2, the latter possibility is the correct one: distinct subtypes have distinct primary sequences arising from distinct genes.

Furthermore, for many excitability phenomena, data have suggested even more diversity within each recognized subtype. Table 3 is a list of instances in which (i) these further distinctions have been rendered more decisive by cloning of multiple structurally related genes for excitability molecules; or (ii) multiple genes have been cloned but there presently exists no functional distinctions among the clones. In the future there will be more examples of such diversity, but the existing catalog may already be complete for some subtypes. For example, in the search for the human β_2-adrenergic receptor gene, both genomic and cDNA libraries have been screened, and three different research groups have identified the same gene.

The Tailored Subtype-Specific Ligand

Excitability proteins have been characterized by cloning and by heterologous expression; the diversity of these proteins arises to a large extent at the genomic level. What are the prospects that more selective new pharmaceuticals can be developed through heterologous expression of genes encoding excitability proteins? The most exciting outcome, which is termed here the "tailored subtype-specific ligand," would result from the following three successes.

1) Neurobiological or physiological studies would identify each subtype of an excitability protein with a functional pathway.

2) These functional distinctions would be correlated with a clinically relevant phenomenon.

3) With selective heterologous expression of each subtype as an assay, ligands would be identified that specifically affect only one

Table 1. Cloned excitability proteins.

Molecule	Effector	Postulated transmembrane structure of subunit	Subunits	References
		Seven-helix receptors		
α_2 adrenergic	G protein	7 helices	1	(*56*)
β_1 adrenergic				(*57–59*)
β_2 adrenergic				(*14, 15, 30, 60, 61*)
M1 muscarinic ACh				(*62–64*)
M2 muscarinic ACh				(*27, 59, 65*)
Serotonin $5HT_{1C}$				(*4, 6, 16*)
Substance K				(*5*)
		Ligand-gated channels		
Nicotinic ACh (muscle)	Channel	4–5 helices	$\alpha_2\beta\gamma\delta$	(*66*)
Nicotinic ACh (nerve)			$\alpha_2\beta_2$ or $\alpha_2\beta_3$	(*67–73*)
$GABA_A$		4 helices	$\alpha_2\beta_2$	(*73a*)
Glycine, 48-kD subunit		4 helices	48, 58, 93 kD	(*74*)
		Voltage-dependent channels		
Na^+	Channel	24 helices	*Electrophorus*: α Mammal:αβ1β2	(*75*) (*35, 36, 76, 77*)
Ca^{2+} (DHP-binding subunit)		α1, 24 helices	α1α2βγ(δ)	(*17, 78*)
A-type K^+		6–7 helices	Unknown	(*18–20, 24*)
		Ion pumps		
Na^+,K^+-ATPase	Pump	α, 6–10 helices β, 1 helix	$\alpha_n\beta_n$	(*79–85*)
H^+,K^+-ATPase		10 helices		(*31*)
Ca^{2+}-ATPase of sarcoplasmic reticulum		10 helices		(*86, 87*)

subtype. Researchers would use expressed human genes rather than animal systems in experiments. This would ensure that the assays accurately represent the drug's action on the intended target tissue.

A project to generate such a tailored subtype-specific ligand for even a single excitability protein would be a major research program spanning many disciplines. Although the challenges of each aspect are great, points 1 and 2 are within the capabilities of modern biology. The rest of this article considers point 3.

Milestones in the Research Program

To obtain decisive functional information in a heterologous expression system, one requires full-length DNAs that code for all of the known subunits of the receptor, channel, or transporter. Furthermore, complete testing of the tailored subtype-specific ligand would require expression of all the subtypes of a particular excitability molecule.

Cloning. The ability to identify appropriate clones and to verify possession of all the subunits of a given functional complex involves successful reconstitution of all known pharmacological and functional properties. For instance, binding studies confirmed the β_2-adrenergic character of receptors expressed from human cDNAs that were originally identified on the basis of hybridization with a cDNA for a hamster β_2-adrenergic receptor (*14, 15*).

Obtaining a complete set of full-length clones will presumably be an achievable goal for the seven-helix family because they consist of a single, relatively small (~450 amino acids) peptide. All known adrenergic and muscarinic ACh receptors lack introns in the coding region, simplifying the process of cloning from genomic libraries (which are more likely than cDNA clones to contain the entire coding region). However, this generalization does not apply to the entire seven-helix family: like the opsins, the mouse $5HT_{1C}$ receptor gene has introns (*16*). Furthermore, for functional and binding information, the receptor must bind to its correct G protein; thus one may have to clone and express an additional three proteins. Alternatively, the cloned gene could be expressed in the proper cell type, perhaps with the native receptor deleted.

For the voltage-dependent channel family, prospects for functional expression vary with individual members. The α subunits of voltage-dependent Na^+ channels are specified by mRNAs with a coding region of ~6000 nucleotides (nt). Present cDNA cloning procedures rarely produce inserts of this length; thus one must identify and ligate partial-length cDNA clones. The coding region for the α_1 subunit of the putative voltage-dependent Ca^{2+} channel from skeletal muscle is just as long as that of the voltage-dependent Na^+ channel; furthermore, the channel contains additional peptides with largely unknown functional importance (*17*). These complexities may explain the absence of reports on functional Ca^{2+} channels produced in oocytes by in vitro–synthesized mRNA for the α_1 subunit alone.

Different concerns apply to K^+ channels. The *Shaker* locus in *Drosophila* produces many mRNAs with coding regions of about 2000 nt. Each coding region seems to represent one of the four homology regions that are in the Na^+ channel or the α_1 subunit of Ca^{2+} channels. The functional A-type K^+ channel itself may constitute a homo- or hetero-oligomer of such peptides. The major uncertainties will therefore concern the particular set of subunits that make up each type of K^+ channel (*18–21*).

Implications of alternative mRNA processing. For a gene consisting of

Table 2. Successful heterologous expression of cloned excitability proteins. Abbreviations: cAMP, adenosine 3′,5′-monophosphate; PI, phosphatidylinositol.

Molecule	Target cell	Signal	References
		Seven-helix receptors	
α_2 adrenergic	*Xenopus* oocyte	Agonist and antagonist binding	(*56*)
β_1 adrenergic	*Xenopus* oocyte	Binding; adenylate cyclase	(*58*)
β_2 adrenergic	Mouse fibroblast L	Binding; adenylate cyclase	(*7*)
	COS-7 (transient)	Binding	(*7, 88*)
	B-82 L	Binding; cAMP increase	(*89*)
	Xenopus oocyte	Binding; adenylate cyclase	(*14*)
	Mouse fibroblast L	Coupling to G_s	(*90*)
	Mouse fibroblast L	Desensitization; sequestration	(*91*)
M1 muscarinic ACh	*Xenopus* oocyte	Binding; Cl^- current	(*62*)
	A9 L	Antagonist binding	(*92*)
		EGTA-sensitive K^+ conductance	(*93*)
	Mature *Xenopus* egg	Activation	(*94*)
M2 muscarinic ACh	*Xenopus* oocyte	Small Cl^- currents; EGTA-insensitive conductance	(*48*)
	CHO	Antagonist binding; cAMP increase; PI hydrolysis	(*27, 49*)
Serotonin $5HT_{1C}$	*Xenopus* oocyte	Cl^- current	(*4,* 6*)
	3T3 fibroblast	Intracellular Ca^{2+} increase	(*6*)
Substance K	*Xenopus* oocyte	Cl^- current	(*5*)
		Ligand-gated channels	
Nicotinic ACh (muscle)	*Xenopus* oocyte	Agonist-induced conductance; immunolabeling; agonist/antagonist binding	(*32, 39, 51, 95*)
	Mouse fibroblast L	Binding; conductance; single channels	(*8*)
	Yeast	Immunolabeling	(*9*)
Nicotinic ACh (nerve)	*Xenopus* oocyte	Agonist-induced depolarization	(*71, 72*)
$GABA_A$	*Xenopus* oocyte	Agonist-induced conductance	(*73a*)
		Voltage-dependent channels	
Na^+	*Xenopus* oocyte	Voltage clamp	(*33–36*)
A-type K^+	*Xenopus* oocyte	Voltage clamp	(*21*)
		Ion pumps	
Na^+,K^+-ATPase	Mouse fibroblast L	Immunolabeling	(*42*)
	CV-1	Ouabain resistance	(*84*)
	Xenopus oocyte	ATPase; Rb flux; ouabain binding	(*40*)

*Hybrid-selected RNA.

Table 3. Further diversity of excitability proteins.

Molecule	Number of genes	Differential expression	References
		Seven-helix receptors	
M1 muscarinic ACh	3	Brain region; pancreas	(*63, 96*)
		Ligand-gated channels	
Nicotinic ACh, γ/ε	2	Development	(*32, 97*)
Nicotinic ACh (nerve)	3	Brain region	(*68, 69, 71, 98*)
		Voltage-gated channels	
Na^+	4	Brain region; brain versus periphery; development	(*36, 76, 99*)
		Ion pumps	
Na^+,K^+-ATPase	3 (rat) 2 to 4 (human)	Tissue; development	(*29, 80, 85*) (*82*)
Ca^{2+}-ATPase	2	Fast- versus slow-twitch, heart	(*87*)

multiple exons, diversity at the amino acid level is sometimes generated by the use of multiple initiation sites for promoters or alternative splicing patterns (*22*). If an excitability molecule is encoded by such a complex transcription unit, then alternative splicing would introduce additional complexities into a project that uses the heterologous expression of entire genes. For such a program, genomic DNA sequences alone are too complex; one must identify and express the mRNA that is synthesized in a specific target tissue.

Transient K^+ channels [A channels (*23*)] may be an excellent example of such a situation in which diverse excitability proteins are generated by alternative splicing. The *Drosophila Shaker* gene, which encodes a subunit of an A channel, is large, more than 100 kb, and contains multiple introns; within this transcription unit there is evidence for quite varied splicing patterns that eventually produce mRNAs with coding regions of about 2000 nt (*24–26*). Ten distinct cDNA clones have been sequenced, and ten additional clones have been characterized by restriction mapping (most cDNAs are derived from mRNA from heads of flies). The data indicate that the *Shaker* transcripts comprise at least three different 5′ end classes (each composed of multiple exons), a common central region containing three of the seven putative transmembrane α helices, and about four different 3′ end classes containing zero to four of the remaining helices (*19, 20, 25, 26*). The mRNA for these transcripts differs in abundance between the head and body regions (*25*), suggesting that tissue-specific regulation of splicing will be found. Functional consequences of this differential splicing are largely unexplored, but recent results show that RNA synthesized from at least three of the alternatively spliced *Shaker* cDNA clones yields A currents with distinguishable kinetics in oocytes (*21*). If, then, these differential splicing mechanisms help to produce the impressive variations in K^+ channel kinetics among different cell types, a successful ligand development project will be based heavily on knowledge about results of mRNA processing in a particular target cell.

There are several examples of diversity caused by alternative splicing or initiation sites in the noncoding region of mRNA for excitability molecules. Mammalian cardiac (M2) muscarinic ACh receptor mRNA includes one of two distinct 5′ untranslated regions (*27*). The β subunit of the rat Na^+,K^+-ATPase displays tissue-specific variability in transcription initiation sites and in polyadenylation sites (*28*). A similar situation may apply to the α_3 subunit (*29*). The human β_2-adrenergic receptor (*30*) and the H^+,K^+-ATPase (*31*) have short upstream open reading frames. The function of such diversity is largely unknown at this point, and it seems unimportant from the general viewpoint of ligand-protein interactions or of effects on signaling by a molecule once it is expressed on the cell surface.

Expression. Nearly everything can be expressed (Table 2). Furthermore, there is generally faithful expression of pharmacological and functional properties. For example, (i) both oocytes and mammalian cell lines express the binding selectivity of M1 versus M2 cholinergic receptors and of β_1- versus β_2-adrenergic seven-helix receptors; and (ii) oocytes reproduce the distinctions between single-channel characteristics of fetal and adult ACh receptors; these distinctions result from alternative inclusion of the ε or γ subunits (*32*).

There are certain exceptions that cannot be expressed. Of the four known distinct rat brain Na^+ channel cDNAs, one fails to give robust functional expression in oocytes (*33–36*). Similarly, little or no Na^+ channel expression is obtained either with mRNA generated from *Electrophorus electricus* Na^+ channel clones or polyadenylated RNA from the electroplax themselves (*33, 37*); thus the probable cause is not a missing subunit but a deficiency in the expression system. Indeed, the expressed *Electrophorus* Na^+ channel protein is incorrectly glycosylated (*38*). There are several other examples of incorrect glycosylation in RNA-injected oocytes, both with excitability molecules (*39, 40*) and with other proteins (*41*). Transfected mouse fibroblast cells may also incorrectly glycosylate *Torpedo* ACh receptor γ subunits (*8*) and Na^+,K^+-ATPase β subunits (*42*).

In addition to the few complete failures of expression, other results are more troubling with regard to the detailed reconstitution of native properties. Oocytes express Na^+ channels with abnormally high tetrodotoxin sensitivity from mammalian heart RNA, possibly another example of incorrect posttranslational processing (*43*). Even when mRNA that encodes a single protein is sufficient to induce a functional Na^+ channel (the α subunit of a rat brain Na^+ channel), Krafte *et al.* (*44*) found that the channel gating differs from that induced by injections of total brain mRNA. Specifically, in this case, the α-subunit mRNA alone gives channels that display abnormally slow macroscopic inactivation. The addition of unidentified lower molecular weight mRNA species produces more rapid, normal inactivation (*36, 44*).

Coupling to second messengers. Seven-helix receptors participate in a complex signal pathway: (i) the agonist activates the receptor; (ii) the receptor activates a G protein; (iii) the G protein in turn activates or inhibits an effector enzyme or an ion channel; (iv) second messengers activate kinases or channels; and (v) possible later events include down-regulation and phosphorylation-induced desensitization of the receptor itself. How much of this pathway must be reconstituted to provide meaningful absolute or relative assessments of the interaction between a candidate ligand and an expressed receptor? Abundant data indicate that ligand binding is affected by the presence and state of the G protein, but most researchers assume that subsequent steps receive only a single graded signal resulting from the agonist-receptor interaction. Therefore,

ligand screening probably can be conducted in cells that express (or can be induced to express) the appropriate G protein, with little regard for subsequent steps. However, it is not always clear which G protein is the appropriate one; and until better molecular identification is available for G proteins, researchers may simply assess an expressing cell's complement of endogenous G proteins by the ability to activate various second-messenger systems. In oocytes, some receptors couple very strongly to phospholipase C because of endogenous pathways (Fig. 1). Expressed β-adrenergic receptors produce a 20-fold increase in agonist-stimulated adenylate cyclase (*14*). It is not clear whether the extent of stimulation is limited by the receptor, by the G protein, or by the cyclase itself. Coupling by means of G proteins to K^+ channels (*45*) or to Ca^{2+} channels has not been systematically investigated in oocytes.

Many ligands activate more than one intracellular messenger, and it will be important to know whether one needs to express simultaneously two or more subtypes. A related question is whether heterologous expression can become a tool to examine the specificity of coupling between seven-helix proteins and G proteins. Expressed M2 muscarinic ACh receptors provide the most extensive data on the strength and variety of coupling to intracellular messengers. In the rodent atrium, activated M2 receptors simultaneously inhibit adenylate cyclase (*46*) and, at higher agonist concentrations, activate phospholipase C (*47*) and K^+ channels. In experiments on *Xenopus* oocytes, the expressed rat M2 muscarinic ACh receptors activated both an EGTA-insensitive nonspecific cation conductance [perhaps through an endogenous G protein, as in (*45*)] and, weakly, phospholipase C (*48*). In Chinese hamster ovary (CHO) cells, transfected rat M2 muscarinic ACh receptors both decreased adenylate cyclase and, at higher concentrations, activated phospholipase C (*49*). As in the source tissue, expressed receptors can thus couple to multiple second-messenger systems, perhaps by means of multiple G proteins.

Prospects for structural information. A systematic approach to drug design is possible only when one knows the three-dimensional structure of the intended binding site. Heterologous expression can provide an improved assay for drugs designed according to the best available structural information; can it also provide a route to such information?

Atomic-scale structural information is not yet available for any excitability molecule. Such determinations will depend on improved techniques for crystallization of intrinsic membrane proteins; they will also require milligram quantities of the excitability protein. The *Torpedo* ACh receptor and the *Electrophorus* Na^+ channel may soon yield crystals suitable for high-resolution x-ray diffraction studies. Nonetheless, for the majority of excitability proteins, the source tissue cannot readily provide the required amount of pure protein. One may therefore ask whether heterologous expression can yield enough material for structural studies. Bacteria and yeast have often been used for expression of proteins for physical studies (*50*), but there are no reports of functional or even intact excitability proteins expressed by bacteria or yeast. With oocyte injection, optimal expression usually amounts to $\sim 10^{10}$ molecules per cell (*51*); for a protein of 100 kD, one would need to inject 10^7 oocytes for 1 mg of excitability protein. This is not reasonable.

However, heterologous expression in mammalian cells can be highly efficient, with reported levels of $>10^6$ muscarinic ACh receptors per stably transfected CHO cell (*49*). The target of 1 mg would therefore require $\sim 10^{10}$ cells, a feasible quantity in this case. Thus, availability of protein is a manageable hurdle in some cases.

Even given the structural information about an excitability molecule, chemical skill and perhaps fundamentally new knowledge will still be required to build a ligand based on such information. Although we can hope that a structural solution for one member of a family will apply approximately to other members as well—for instance, ACh and GABA receptors probably have the same transmembrane topology—detailed high-resolution data will be required for each ligand binding site.

Prospects

Heterologous expression will have several applications for the study of excitability molecules (*52, 53*) and could help to overcome two major problems that necessitate our present highly empirical approach to the design of drugs that act on different subtypes of excitability proteins: (i) lack of adequate functional assays for the individual subtypes; and (ii) lack of structural information about the ligand-protein interaction. Present chances for success are greatest for the seven-helix family, with its small single-peptide receptors, existing catalog of synthetic high-affinity ligands, and multiplicity of subtypes. At the other end of the spectrum would be excitability molecules with unexplored molecular biology, such as the receptors for excitatory amino acids.

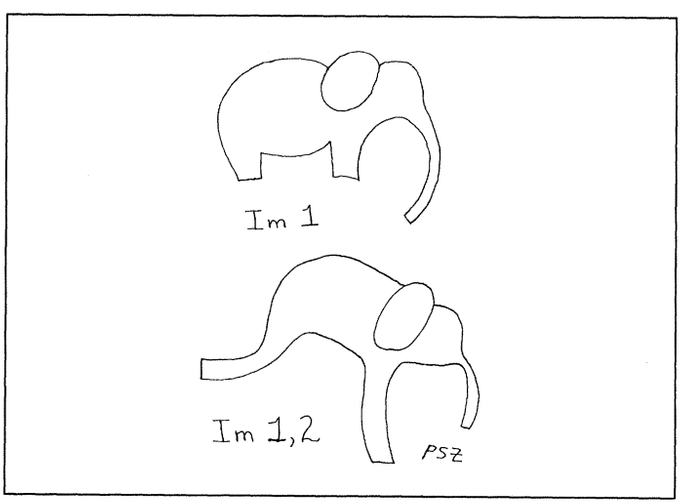
Perhaps the most dramatic result of the development process outlined here would be the emergence of tailored subtype-specific ligands for molecules previously thought to be so widespread and crucial that nonspecific effects would render any intervention useless. Possible examples are (i) the excitatory amino acid receptors and (ii) voltage-dependent K^+ channels. The latter certainly govern impulse-firing frequencies in many neurons and in cardiac muscle; yet study of the clinical pharmacology of these channels has only just begun (*54*). Success in one of these areas would be the latest step in the pharmacological advances that began with Dale's distinction between nicotinic and muscarinic effects of ACh (*55*). Furthermore, the research program would generate exciting progress in physiology, neurobiology, and molecular biology.

REFERENCES AND NOTES

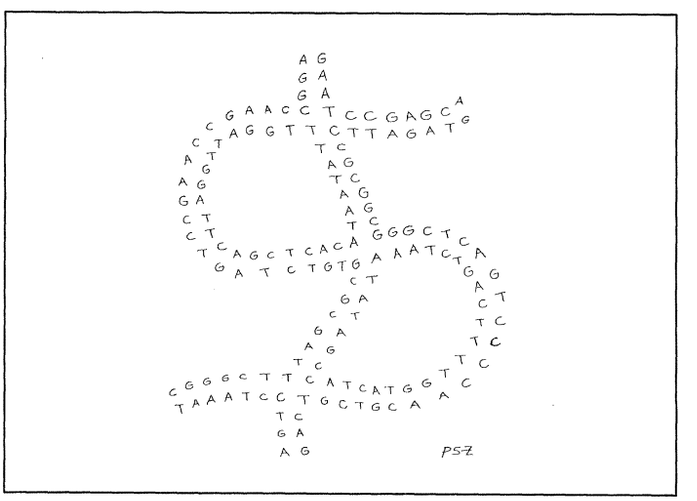
1. K. J. Sweadner, *J. Biol. Chem.* **254**, 6060 (1979).
2. F. Ismail-Beigi, I. S. Edelman, G. G. Gick, *Clin. Res.* **36**, A542 (1988).
3. F. Ashcroft, *Annu. Rev. Neurosci.* **11**, 97 (1988).
4. H. Lübbert *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4332 (1987).
5. Y. Masu *et al.*, *Nature* **329**, 836 (1987).
6. D. Julius, A. B. MacDermott, R. Axel, T. M. Jessell, *Science* **241**, 558 (1988).
7. R. A. F. Dixon *et al.*, *EMBO J.* **6**, 3269 (1987).
8. T. Claudio *et al.*, *Science* **238**, 1688 (1987).
9. N. Fujita *et al.*, *ibid.* **231**, 1284 (1986).
10. B. Cullen, *Methods Enzymol.* **152**, 684 (1987).
11. N. M. Nathanson, *Annu. Rev. Neurosci.* **10**, 195 (1987); H. Ladinsky *et al.*, *Trends Pharmacol. Sci. Suppl: Subtypes of Muscarinic Receptors* (1988), p. 44. DNA sequence data have demonstrated there are at least four muscarinic receptor genes (Table 3).
12. M. C. Nowycky, A. P. Fox, R. W. Tsien, *Nature* **316**, 440 (1985).
13. D. M. Fambrough and E. K. Bayne, *J. Biol. Chem.* **258**, 3926 (1983); W. L. Stahl, *Neurochem. Int.* **8**, 449 (1986).
14. B. K. Kobilka *et al.*, *J. Biol. Chem.* **262**, 15796 (1987).
15. F. Z. Chung *et al.*, *FEBS Lett.* **211**, 200 (1987).
16. B. Hoffman, thesis, John Hopkins University (1988).
17. M. Takahashi, M. J. Seagar, J. F. Jones, B. F. X. Reber, W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5478 (1987); P. L. Vaghy *et al.*, *J. Biol. Chem.* **262**, 14337 (1987); N. Nakayama, T. Kirley, P. Vaghy, E. McKenna, A. Schwartz, *ibid.*, p. 6572; J. Striessnig *et al.*, *FEBS Lett.* **212**, 247 (1987); A. T. Leung, T. Imagawa, K. P. Campbell, *J. Biol. Chem.* **262**, 7943 (1987); A. T. Leung, T. Imagawa, B. Block, C. Franzini-Armstrong, K. P. Campbell, *ibid.* **263**, 994 (1988).
18. B. L. Tempel, D. M. Papazian, T. L. Schwarz, Y. N. Jan, L. Y. Jan, *Science* **237**, 770 (1987).
19. D. M. Papazian, T. L. Schwarz, B. L. Tempel, Y. N. Jan, L. Y. Jan, *ibid.* p. 749.
20. A. Kamb, L. E. Iverson, M. A. Tanouye, *Cell* **50**, 405 (1987).
21. L. C. Timpe *et al.*, *Nature* **331**, 143 (1988); L. Iverson, M. A. Tanouye, H. A. Lester, N. Davidson, B. Rudy, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
22. S. E. Leff, M. G. Rosenfeld, R. M. Evans, *Annu. Rev. Biochem.* **55**, 1091 (1986); E. M. Jorgensen and R. L. Garber, *Genes Dev.* **1**, 554 (1987).
23. J. A. Connor and C. F. Stevens, *J. Physiol.* **213**, 1 (1971); *ibid.*, p. 21; *ibid.*, p. 31; S. Hagiwara, K. Kusano, N. Saito, *ibid.* **155**, 470 (1961); E. Neher, *J. Gen. Physiol.* **58**, 36 (1971).
24. A. Baumann *et al.*, *EMBO J.* **6**, 3419 (1987).

25. T. L. Schwarz, B. L. Tempel, D. M. Papazian, Y. N. Jan, L. Y. Jan, *Nature* **331**, 137 (1988).
26. A. Kamb, J. Tseng-Crank, M. A. Tanouye, *Neuron*, in press.
27. E. G. Peralta *et al.*, *Science* **236**, 600 (1987).
28. R. M. Young, G. E. Shull, J. B. Lingrel, *J. Biol. Chem.* **262**, 4905 (1987).
29. V. L. Herrera, J. R. Emanuel, N. Ruiz-Opazo, R. Levenson, G. Nadal-Ginard, *J. Cell Biol.* **105**, 1855 (1987).
30. B. K. Kobilka *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 46 (1987).
31. G. E. Shull and J. B. Lingrel, *J. Biol. Chem.* **261**, 16788 (1986).
32. M. Mishina *et al.*, *Nature* **321**, 406 (1986).
33. M. Noda *et al.*, *ibid.* **322**, 826 (1986).
34. W. Stühmer, C. Methfessel, B. Sakmann, M. Noda, S. Numa, *Eur. Biophys. J.* **14**, 131 (1987).
35. T. Kayano *et al.*, *FEBS Lett.* **228**, 187 (1988).
36. V. Auld *et al.*, *Neuron*, in press.
37. C. Hirono *et al.*, *Brain Res.* **359**, 57 (1985).
38. W. B. Thornhill and S. R. Levinson, *Biochemistry* **26**, 4381 (1987).
39. M. Mishina *et al.*, *Nature* **313**, 364 (1985).
40. S. Noguchi *et al.*, *FEBS Lett.* **225**, 27 (1987).
41. H. Soreq, *CRC Crit. Rev. Biochem.* **18**, 199 (1985).
42. K. Takeyasu *et al.*, *J. Biol. Chem.* **262**, 10733 (1987).
43. F. Sutton, N. Davidson, H. A. Lester, *Mol. Brain Res.* **3**, 187 (1987).
44. D. A. Krafte, T. P. Snutch, J. P. Leonard, N. Davidson, H. A. Lester, *J. Neurosci.*, in press.
45. P. J. Pfaffinger, J. M. Martin, D. D. Hunter, N. M. Nathanson, B. Hille, *Nature* **317**, 536 (1985).
46. J. H. Brown, *Mol. Pharmacol.* **16**, 841 (1981).
47. S. L. Brown and J. H. Brown, *ibid.* **24**, 351 (1983).
48. K. Fukuda *et al.*, *Nature* **327**, 623 (1987).
49. A. Ashkenazi *et al.*, *Science* **238**, 672 (1987).
50. A. R. Shatzman and M. Rosenberg, *Methods Enzymol.* **152**, 661 (1987); G. A. Bitter, *ibid.*, p. 673.
51. B. Sakmann *et al.*, *Nature* **318**, 538 (1985); K. Yoshii, L. Yu, K. Mixter Mayne, N. Davidson, H. A. Lester, *J. Gen. Physiol.* **90**, 553 (1987).
52. N. Dascal, *CRC Crit. Rev. Biochem.* **22**, 317 (1987).
53. T. Snutch, *Trends Neurosci.* **11**, 250 (1988).
54. N. Taira, *J. Cardiovasc. Pharmacol.* **10**, S1 (1987); K. Sumimoto *et al.*, *ibid.*, p. S66; S. W. Weir and A. H. Weston, *Br. J. Pharmacol.* **88**, 113 (1986); T. C. Hamilton, S. W. Weir, A. H. Weston, *ibid.*, p. 103.
55. H. H. Dale, *J. Pharmacol. Exp. Ther.* **6**, 147 (1914).
56. B. K. Kobilka *et al.*, *Science* **238**, 650 (1987).
57. Y. Yarden *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6795 (1986).
58. T. Frielle *et al.*, *ibid.* **84**, 7920 (1987).
59. J. Gocayne *et al.*, *ibid.*, p. 8296.
60. R. A. F. Dixon *et al.*, *Nature* **321**, 75 (1986).
61. P. R. Schofield, L. M. Rhee, E. G. Peralta, *Nucleic Acids Res.* **15**, 3636 (1987).
62. T. Kubo *et al.*, *Nature* **323**, 411 (1986).
63. T. I. Bonner, N. J. Buckley, A. C. Young, M. R. Brann, *Science* **237**, 527 (1987).
64. T. Braun, P. R. Schofield, B. D. Shivers, D. B. Pritchett, P. H. Seeburg, *Biochem. Biophys. Res. Commun.* **149**, 125 (1987).
65. T. Kubo *et al.*, *FEBS Lett.* **209**, 367 (1986).
66. M. Noda *et al.*, *Nature* **302**, 528 (1983); R. M. Stroud and J. Finer-Moore, *Annu. Rev. Cell. Biol.* **1**, 317 (1985).
67. P. Nef, C. Oneyser, T. Barkas, M. Ballivet, in *Nicotinic Acetylcholine Receptor: Structure and Function*, A. Maelicke, Ed. (Springer-Verlag, New York, 1986), pp. 417–422.
68. J. Boulter *et al.*, *Nature* **319**, 368 (1986).
69. J. Boulter *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7763 (1987).
70. J. Lindstrom, R. Schoepfer, P. Whiting, *Mol. Neurobiol.* **1**, 281 (1987).
71. K. Wada *et al.*, *Science* **240**, 330 (1988).
72. E. S. Deneris *et al.*, *Neuron* **1**, 45 (1988).
73. R. Schoepfer *et al.*, *ibid.*, p. 241.
73a. P. R. Schofield *et al. Nature* **328**, 221 (1987).
74. G. Grenningloh *et al.*, *ibid.* **328**, 215 (1987).
75. M. Noda *et al.*, *ibid.* **312**, 121 (1984).
76. M. Noda *et al.*, *ibid.* **320**, 188 (1986).
77. A. L. Goldin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7503 (1986); L. Salkoff *et al.*, *Science* **237**, 744 (1987).
78. T. Tanabe *et al.*, *Nature* **328**, 313 (1987).
79. G. E. Shull, A. Schwartz, J. B. Lingrel, *ibid.* **316**, 691 (1985).
80. G. E. Shull, J. Greeb, J. B. Lingrel, *Biochemistry* **25**, 8125 (1986).
81. G. E. Shull, L. K. Lane, J. B. Lingrel, *Nature* **321**, 429 (1986).
82. M. M. Shull and J. B. Lingrel, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4039 (1987).
83. K. Kawakami *et al.*, *Nature* **316**, 733 (1985); K. Kawakami, H. Nojima, T. Ohta, K. Nagano, *Nucleic Acids Res.* **14**, 2833 (1986); K. Kawakami *et al.*, *J. Biochem.* **100**, 389 (1986); Y. A. Ovchinnikov *et al.*, *Dokl. Akad. Nauk SSSR* **285**, 1490 (1985); Y. A. Ovchinnikov *et al.*, *ibid.* **287**, 1491 (1986); Y. A. Ovchinnikov *et al.*, *FEBS Lett.* **201**, 237 (1986); S. Noguchi *et al.*, *ibid.* **225**, 27 (1986); R. W. Mercer *et al.*, *Mol. Cell. Biol.* **6**, 3884 (1986).
84. R. B. Kent, J. R. Emanuel, Y. B. Neriah, R. Levenson, D. E. Housman, *Science* **237**, 901 (1987).
85. R. B. Kent *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5369 (1987).
86. D. H. MacLennan *et al.*, *Nature* **316**, 696 (1985).
87. C. J. Brandl *et al.*, *Cell* **44**, 597 (1986).
88. R. A. F. Dixon *et al.*, *Nature* **326**, 73 (1987).
89. C. M. Fraser, F. Z. Chung, J. C. Venter, *J. Biol. Chem.* **262**, 14843 (1987).
90. C. D. Strader *et al.*, *ibid.*, p. 16439.
91. C. D. Strader *et al.*, *Cell* **49**, 855 (1987).
92. M. R. Brann, N. J. Buckley, S. V. Jones, T. I. Bonner, *Mol. Pharmacol.* **32**, 450 (1987).
93. S. V. P. Jones, J. L. Barker, T. I. Bonner, N. J. Buckley, M. R. Brann, *Biophys. J.* **53**, 636a (1988).
94. D. Kline *et al.*, *Science* **241**, 464 (1988).
95. M. Mishina *et al.*, *Nature* **307**, 604 (1984); M. M. White, K. Mixter Mayne, H. A. Lester, N. Davidson, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4852 (1985); T. Claudio, *ibid.* **84**, 1567 (1987).
96. E. Peralta *et al.*, *EMBO J.* **6**, 3923 (1987).
97. V. Witzemann, B. Barg, Y. Nishikawa, B. Sakmann, S. Numa, *FEBS Lett.* **223**, 104 (1987).
98. D. Goldman, D. Simmons, L. W. Swanson, J. Patrick, S. Heinemann, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 4076 (1986); D. Goldman *et al.*, *Cell* **48**, 965 (1987).
99. D. Gordon *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8682 (1987); S. S. Cooperman *et al.*, *ibid.*, p. 8721.
100. W. B. Busa and R. Nuccitelli, *J. Cell Biol.* **100**, 1325 (1985); W. Busa, J. E. Ferguson, S. K. Joseph, J. R. Williamson, R. Nuccitelli, *ibid.* **101**, 677 (1985).
101. L. A. Jaffe and N. L. Cross, *Annu. Rev. Physiol.* **48**, 191 (1986); D. Kline, *Dev. Biol.* **126**, 346 (1988).
102. C. B. Gundersen, R. Miledi, I. Parker, *Proc. R. Soc. London B* **219**, 103 (1983); N. Dascal *et al.*, *Mol. Brain Res.* **1**, 201 (1986); Y. Nomura, S. Kaneko, K. Kato, S. Yamagishi, H. Sugiyama, *ibid.* **2**, 113 (1987); T. Takahashi, E. Neher, B. Sakmann, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5063 (1987).

103. I thank N. Davidson, T. M. Fong, H. Graves, D. S. Krafte, L. A. Jaffe, D. Julius, D. Kline, W. Koch, C. Labarca, R. J. Leonard, J. Lindstrom, E. McKenna, R. W. Olsen, J. H. Sabry, A. Schwartz, M. Tanouye, and L. Yu for comments on this article and M. A. Buckles for assistance with the manuscript. Supported by grants from NIH, the Klingenstein Fund, and the Drown Foundation.



News note: Unusually shaped antibodies have been discovered that cause water molecules to "remember."



News item: A third genetic code has been discovered that translates nucleotide sequences into cold, hard cash.