J. Membrane Biol. 126, 97–108 (1992)

The Journal of
Membrane Biology

Topical Review

Heterologous Expression of Calcium Channels

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Introduction

Calcium entry through voltage-dependent Ca channels triggers various cellular functions including excitation-contraction coupling in muscles, secretion of hormones by endocrine cells and release of neurotransmitters by neurons. Patch-clamp studies have revealed the existence of multiple types of Ca channels; classification schemes are based on their electrophysiological properties and their specific pharmacology. According to one such scheme, the T type is activated at rather negative potentials and no specific blocker has yet been reported; the L type is activated at less negative potentials and is sensitive to dihydropyridines (DHP) and other Ca antagonists. L and T types are widely distributed and coexist in a large variety of cells (reviewed by Bean, 1989). On the other hand, N-type Ca channels (Nowycky, Fox & Tsien, 1985) have exclusively been found in neuronal cells and are blocked by the ω-toxin from *Conus geographicus* (ω-CgTx) (McCleskey et al., 1987). P-type channels, whose existence was suggested by Llinas, Sugimori and Cherksey (1989) in Purkinje cells, are insensitive to (ω-CgTx) but blocked by a funnel web spider toxin (FTX; Lin, Rudy & Llinas, 1990). Several reviews on Ca channels have appeared recently (Hess, 1990; Miller & Fox, 1990; Tsien & Tsien, 1990).

Because of the availability of specific ligands such as dihydropyridines and a rich source of DHP binding sites in T tubule membranes of skeletal muscle, the Ca channel protein has been purified by several groups (Borsetto et al., 1984; Curtis & Catterall, 1984; Leung, Imagawa & Campbell, 1987). It is now accepted that the Ca channel protein is constituted of five subunits ($\alpha 1, \alpha 2/\delta, \beta, \gamma$), the major subunit $\alpha 1$ including both the channel and the binding sites for Ca antagonists (for review, Vaghy et al., 1987, 1988; Campbell, Leung & Sharp, 1988; Catterall, 1988; Hosey & Lazdunski, 1988; McKenna et al., 1990). The $\alpha 1\alpha 2$ complex, probably including β, γ, and δ as well, is found in most tissues (Takahashi & Catterall, 1987; Ahlijanian, Westenbroek & Catterall, 1990), but structural differences exist, for instance in the molecular weight of $\alpha 1$ among tissues (Chang & Hosey, 1988; Schneider & Hofmann, 1988; Yoshida et al., 1990).

Molecular biological studies have revealed the primary sequences of the $\alpha 1$ subunit of skeletal muscle (Tanabe et al., 1987; Ellis et al., 1988; Grabner et al., 1991), heart (Mikami et al., 1989), pulmonary smooth muscle (Biel et al., 1990), and vascular smooth muscle (Koch, Ellinor & Schwartz, 1990). Partial sequences have also been reported for several $\alpha 1$ subunits from brain (Koch et al., 1989; Snutch et al., 1990), and confirmatory full length sequence for two of these subunits were recently published (Hui et al., 1991; Mori et al., 1991; Snutch et al., 1991).

From these studies it seems clear that several genes, and additionally tissue specific isoforms arising from alternative splicing, are responsible for the diversity of Ca channels. How does the molecular diversity relate to functional and pharmacological properties? Expression of mRNA or DNA in foreign cells has been proven to be a very useful form of functional reconstitution and is termed heterologous expression. The favorite expression system is based on the injection of mRNA in *Xenopus* oocytes. Such expression serves both as a decisive proof of successful cloning and as a tool for studying the structure and function of Ca channels expressed from

Key Words oocytes · transfected mammalian cells · cardiac muscle · skeletal muscle · brain

various tissues after injection of total or poly(A+) mRNA. This system, together with emerging techniques of expression in mammalian cells, now offers an attractive way to study the role of the different subunits of Ca channels by coinjection of mRNA transcribed in vitro for cDNA coding from $\alpha 1$, $\alpha 2$, β or γ.

Voltage-Dependent Ca Channels From Various Tissues Can Be Expressed in *Xenopus* Oocytes

Xenopus oocytes have been used to express foreign proteins from mRNA injection in the cytoplasm since the work of Gurdon et al. (1971). The first successful expression of plasma membrane proteins was reported 10 years later, when the presence and functional expression of nicotinic actylcholine receptors was demonstrated in oocyte membranes following the injection of crude mRNA isolated from *Torpedo* electrical organ. (Sumikawa et al., 1981). This was rapidly followed by reports on expression of other ion channels and receptors, particularly by Miledi and colleagues (reviewed by Lester, 1988; Sigel, 1990). One feature of the membrane proteins expressed after microinjection of crude mRNA was the apparent conservation of the pharmacological and electrophysiological properties observed in the origin tissue. It is particularly striking that the *Xenopus* oocyte can reassemble membrane proteins consisting of several polypeptides after coinjection of the in vitro synthesized mRNA encoding each subunit. *Xenopus* oocytes do not, however, seem able to express all membrane proteins. Until recently it was thought that oocytes were incapable of robust Ca channel expression, but it is now apparent that excellent expression is indeed obtained when all appropriate subunit RNAs are injected.

Oocytes Have Endogenous Ca Channels and Ca-Activated Cl Channels

Functional detection of newly expressed Ca channels can be limited by endogenous or exogenous conductances. The presence of several voltage-dependent ion channels has been reported on the denuded oocytes generally used in these experiments. As a precaution against contamination by the endogenous voltage-dependent Na^+ channels found in a few oocytes and induced by RNA from several tissues, tetrodotoxin is generally included in the bathing solution in experiments conducted on Ca channels. A slow endogenous Na current was also described in some batches of oocytes for strong depolarizations (Baud, Kado & Marcher, 1982). Care must also be taken to suppress endogenous and RNA-induced voltage-dependent K currents. Ca channels in oocytes are usually studied using Ba^{2+} as the charge carrier for several reasons: (i) Inactivation of Ca channels by Ba^{2+} is minimal, (ii) Ba^{2+} does not efficiently activate the Ca^{2+}-dependent Cl^- current described below, (iii) K^+ current is largely blocked in Ba^{2+}. Moreover, the K^+ currents can be almost completely suppressed by soaking the injected oocytes 24 hours before recording in a Ringer solution containing 2 mM Cs^+ instead of K^+ (Leonard et al., 1987). This treatment leads, probably via the Na/K pump, to a loading of the intracellular medium with Cs ions but does not affect the survival of the oocyte or the expression of exogenous proteins.

The most prominent endogenous conductance is the Ca^{2+}-dependent Cl^- current, which can be either a limitation in the use of oocytes because it forces one to study Ca channels in the absence of extracellular Ca^{2+}, or an advantage since the Ca-activated chloride current can be used to assay for the presence of Ca channels in a normal Ca-containing external solution. Ca channel activity is directly recorded in most cases in a Ba-methanesulfonate (Ba-MS) solution which suppresses Cl^- currents both (i) because Ba does not activate them (Barish, 1983) and (ii) because methanesulfonate does not permeate through them (Dascal et al., 1986). In addition, Ba currents can be easily separated from residual K^+ currents by subtracting traces in the presence of an inorganic Ca channel blocker such as Cd^{2+} or Co^{2+} (Dascal et al., 1986; Leonard et al., 1987). Although Ca-dependent Cl channels can be almost completely blocked by drugs such as 9-AC, this procedure has not allowed recording of Ca currents (Leonard et al., 1987).

Endogenous Ca channels exist in noninjected oocytes and have been recorded directly by Dascal et al. (1986), Leonard et al. (1987), and Moorman et al. (1987) in 40 mM Ba^{2+}. Ba currents are on the order of 30 nA in a peak amplitude; they activate around -25 mV and peak around $+10$ mV and are transient with an exponential decay time constant of roughly 100 msec. They are totally blocked by $[Cd^{2+}] < 500$ μM but are insensitive to organic Ca channel blockers. A detailed pharmacological analysis of the endogenous Ba current in the presence of several inorganic Ca channel blockers has shown that it is more strongly blocked by Cd^{2+} than by Mn^{2+} (Lory et al., 1990).

Brain RNA Directs the Highest Ca Channel Expression

The expression of Ca channels has been obtained after the microinjection of crude or poly(A+)

mRNA from young (2 to 4 weeks) rat brain (Dascal et al., 1986; Leonard et al., 1987; Kaneko & Nomura, 1987), human brain (Gundersen, Umbach & Swartz, 1988), and *Torpedo* electric lobe (Umbach & Gundersen, 1987). Ba currents were between 200 and 800 nA, ie., 10 to 40 times larger than control Ba currents. In such conditions, Ba current undergoes relatively little inactivation for short depolarizations (300 msec). For longer pulses, more than 80% of the inactivation time course is described by a single time constant of ~600 msec (Leonard et al., 1987). I_{Ba} is activated at potentials more positive than −40 to −30 mV, and peak currents are elicited near +15 mV. There is roughly 50% inactivation of I_{Ba} for a 5 sec conditioning prepulse at −20 mV. Very similar electrophysiological properties were reported for human brain or *Torpedo* electric lobe directed Ca channels (Gundersen et al., 1988; Umbach & Gundersen, 1987). Gundersen et al. (1988) confirmed the exogenous nature of the Ba currents by using actinomycin D (50 mg/ml), an inhibitor of RNA transcription, which does not affect the expression of these currents.

In all these studies, the Ca channels induced by mRNA from a nervous tissue and studied in 40 mM Ba share an insensitivity to the classic organic Ca channel effectors and particularly dihydropyridines. Interestingly, although rat brain directed Ca channels have been reported as insensitive to ω-CgTx, both *Torpedo* electric lobe RNA and human brain RNA direct Ba currents that are blocked under the same experimental conditions by this toxin (1 to 10 μM). Thus, *Torpedo* electric lobe and human temporal cortex RNA encode a channel whose pharmacology differs from that of the dominant channel encoded by RNA from total rat brain. Recent results (Lin et al., 1990) show that the rat brain directed Ca channels are blocked by a fraction from FTX. FTX also blocks the Ca channels in the squid giant synapse and calcium spikes in cerebellar Purkinje cells (Llinas et al., 1989) which are insensitive to both DHP and ω-CgTx and have been designated as P-type. A a result, the calcium channels expressed from rat brain mRNA in *Xenopus* oocytes are suggested to represent P-type Ca channels (Lin et al., 1990).

Another interesting point of the study by Lin et al. (1990) is that the blockade of Ca channels by FTX decreases at higher external Ba concentration. Similar results were reported for the action of DHP on cardiac-directed Ca channels (Lory et al., 1990). Experiments on Ba currents expressed from rat brain mRNA and recorded in 2 mM external Ba have shown a small but significant decrease in the presence of ω-CgTx; this effect could be masked when high concentrations of external Ba are used (P. Lory et al., *unpublished*). These results, taken together with the report of Kaneko and Nomura (1987), would indicate that although a dominant type of Ca channel is expressed from rat brain mRNA, it is likely that a small proportion of L-type and N-type channels are also expressed. Later cDNA cloning data, summarized below, support the suggestion that several related genes encode distinct Ca channel types.

In addition to the use of specific drugs or toxins to characterize brain Ca channels expressed in oocytes, other modulations of the exogenous Ba currents have been reported. Gundersen et al. (1988) have shown that human temporal cortex calcium channels studied in oocytes are depressed by barbituarates which are known to inhibit the release of neurotransmitters in several neuronal preparations. The action of barbiturates is characterized by (i) reduced Ba currents but no change in the current-voltage relationship, (ii) accelerated inactivation, and (iii) a leftward shift in the steady-state inactivation relation. These results, taken together with the high sensitivity of these channels to ω-CgTx, would suggest that the dominant Ca channels expressed in oocytes by human temporal cortex mRNA are similar to N type, although surprisingly low sensitivity to Cd^{2+} is reported.

Brain Ca channels in native cells are also modulated by intracellular messengers involved in the activation of protein kinases. Regulation by A and C kinase has been investigated on rat brain directed Ca channels. While Leonard et al. (1987) found no significant effect on Ba current amplitude in the presence of forskolin, Kaneko and Nomura (1987) have reported an increase of the Ba current following the application of isoproterenol, forskolin or the intracellular injection of cAMP. Such differences may be explained by the fact that oocytes have a high intracellular basal cAMP concentration (Sadler & Maller, 1987) which may vary among batches and/or by the fact that forskolin (and perhaps cAMP) decreases contaminating K currents.

Muscle Displays a Spectrum of Ca Channel Physiology

The common property of the several types of muscles is their ability to develop a contractile response induced by a transient rise of intracellular Ca^{2+} concentration. However, the source of Ca varies among muscle types. In mammalian skeletal muscle, contraction is maintained in the absence of extracellular Ca^{2+} (Armstrong, Bezanilla & Horowicz, 1972); and the release of Ca^{2+} from the sarcoplasmic reticulum is controlled by the DHP receptor of the transverse tubule membrane, where it mainly serves as a volt-

age sensor (Schneider & Chandler, 1973; Rios & Brum, 1987; Tanabe et al., 1987). By contrast, heart and smooth muscle contractions are suppressed when extracellular Ca^{2+} ions are removed, and early electrophysiological studies demonstrated the role of voltage-dependent Ca^{2+} channels in excitation-contraction coupling (Leoty & Raymond, 1972; Vassort & Rougier, 1972; Mironneau, 1973). Ca channels from several muscle types have been expressed in oocytes by means of mRNA injection and their properties compared with those measured in native cells.

Cardiac Ca Channels Display Native Properties When Expressed in Oocytes

Ca channels have been extensively studied in cardiac cells. Two types of Ca channels have been described: an L-type channel found in all cardiac preparations and, mainly in some atrial or sinoatrial cells, an additional T-type channel (for review *see* Pelzer, Pelzer & McDonald, 1990). Dascal et al. (1986) first expressed channels from rat heart mRNA in oocytes. They found a component that was modulated by dihydropyridine antagonists, isoproterenol, acetylcholine and intracellular cAMP and should correspond to the well-characterized L-type Ca channels described in native cells. Dascal et al. (1986) also detected an additional, transient component that could correspond to a T-like channel. However, no subsequent study has provided convincing evidence that the transient component is different from the endogenous Ca channels. Moorman et al. (1987) also studied the Ca channels expressed in oocytes from total adult rat heart mRNA. The expressed Ba currents were rather small—roughly twice the size of endogenous Ba currents. Despite this low expression of exogenous Ca channels, Moorman et al. (1987) were successful in recording single channel activity in 110 mM external Ba. The single-channel properties, i.e., conductance (18–25 pS), activation threshold (−20 to −10 mV), extrapolated reversal potential, bursting behavior, evidence for two open states, and long openings in the presence of Bay K 8644, agree with results on single Ca channels in native tissue.

A detailed study has recently been devoted to the electrophysiological and pharmacological properties of Ca channels expressed in oocytes after microinjection of total or poly(A^+) mRNA from ten day old rats hearts (Lory et al., 1990). It was found that the poly(A^+)-mRNA purification step is essential for enriching the Ca channel message, leading to a three to four-fold increase of the expressed Ba current. This increase enables one to analyze the properties of Ca channels in the presence of lower concentrations of divalent ions and particularly in 2 mM external Ba. In these conditions, the endogenous Ca current is negligible in all batches of oocytes tested, and the Ba current represents exclusively the expressed cardiac Ca current. Moreover, the properties of the expressed Ca current are modified when the external Ba concentration is reduced to 2 mM: there is a leftward shift (~20 mV) of the current-voltage relationship. Activation, peak current and reversal potential are respectively −35, −14 and 26 mV, these values being quite similar to those observed on single rat ventricular cells (Richard et al., 1990). The expressed Ba current exhibits only a partial inactivation, as confirmed by the steady-state inactivation relationship (using a 5-sec prepulse) which also is shifted toward more negative values when the external concentration of Ba is decreased from 40 to 2 mM. While the Ba current is only partly reduced by a high concentration (10 μM) of DHP antagonist (nifedipine or PN 200-110) in 40 mM Ba, it is almost completely suppressed by the same concentration in 2 mM Ba. The importance of the external divalent concentration for the pharmacology of Ca channels was investigated; when external Ba^{2+} was decreased to 2 from 40 mM, there was (i) a clear voltage dependence of the effect of Bay K 8644 on Ba currents illustrated by a leftward shift of the *I/V* curve, (ii) a greater potentiation of Ba currents for negative potentials (between −25 and −30 mV), and (iii) an ~20-fold increase by Bay K 8644.

Taken together, these results of different groups indicate that L-type Ca channels expressed from rat heart have electrophysiological properties and are regulated by dihydropyridines and by β-adrenergic agonists similarly to those observed in the native ventricular cells. The only difference reported by Lory et al. (1990) is a slowing of the Ba current decay in the presence of Bay K 8644. Such a result may be interpreted in terms of an interaction between intracellular cAMP and Bay K 8644 as recently shown on native Ca currents in rat ventricular cells (Tiaho et al., 1990), since the basal cAMP level is higher in oocytes (Sadler & Maller, 1987). One important feature in these experiments is the stability of the Ba currents expressed in oocytes and the absence of rundown. Taking advantage of these properties and of the possibility of microinjection during electrophysiology, recent experiments have been conducted to investigate details of the modulation of expressed Ca channels by PKA and PKC (Lory et al., 1990; Bourinet et al., 1991[1]). These

[1] Bourinet, E., Fournier, F., Lory, P., Charnet, P., Nargeot, J. 1991. (*Submitted*)

studies were conducted using microinjection of cAMP, addition of PMA, and microinjection of peptide inhibitors of kinases A and C, A-PKI (Fernandez et al., 1990) and C-PKI (House & Kemp, 1987), respectively. Results show that A-PKI inhibits the cAMP induced increase of I_{Ba} and also reduces the basal current which, in this state, is only slightly increased by Bay K 8644. This could arise because phosphorylation of Ca channels may be required for activation via Bay K 8644, as suggested for GH3 cells by Armstrong and Eckert (1987). On the other hand, application of the phorbol ester PMA induces a transient increase of the expressed Ba current. After 10 to 20 min, Ba current is progressively reduced to less than the control basal value and can be amost completely eliminated after 30 min. This effect is prevented by prior injection of C-PKI, confirming the role of PKC. An increase by PMA was also observed in the case of rat brain directed Ba currents (Leonard et al., 1987), and later unpublished experiments reveal that this effect is similarly transient.

Skeletal Muscle Calcium Channels Express Poorly or Not At All In Oocytes

T tubule membranes are a rich source of DHP receptor sites (Fosset et al., 1983; Curtis & Catterall, 1984). The consensus is (i) that DHP receptors in skeletal muscle act as voltage sensors (Tanabe et al., 1987), and (ii) that only a small fraction corresponds to functional Ca channels (Schwartz, McCleskey & Almers, 1985). In the first paper on heterologous expression of Ca channels (Dascal et al., 1986), it was reported that skeletal muscle mRNA injected into oocytes leads to the production of Ca channels in the oocyte membrane. In later work, N. Dascal, I. Lotan, E. Karni and A. Gigi (*unpublished*) have found that rat skeletal muscle mRNA induces a transient, Ni^{2+}-sensitive component of Ba^{2+} current and a maintained Ni^{2+}-resistant component. Both components are small (<250 nA) and neither is sensitive to DHP or verapamil. Antisense nucleotides against the SkM $\alpha 1$ subunit failed to suppress either component. These observations render it unlikely that the oocytes are expressing L-type channels from skeletal muscle $\alpha 1$ subunits. It appears likely that the induced currents correspond to the enhanced expression of an endogenous oocyte Ca channel.

In contrast to vertebrate skeletal muscles, invertebrate skeletal muscles from many animals require external calcium for contraction and the presence of Ca channels in the sarcolemmal membrane has been demonstrated (Hencek & Zackar, 1977; Jdaiaa & Guilbaud, 1986). The purification of a DHP receptor from crustacean fibers has been recently reported (Krizanova, Novotova & Zachar, 1990). Total RNA was extracted from crab *Carcinus meanas* leg muscle and poly (A^+) mRNA was obtained as usual and injected in oocytes (Fournier et al., 1990). Injected oocytes exhibit both larger depolarization-evoked Cl^- currents and Ba currents related to exogenous Ca channels. The authors describe a total Ba current of <100 nA activated at relatively negative potentials with a peak activation near 10 mV. These results are interpreted in terms of two Ba currents: a low threshold transient current insensitive to DHP and a maintained high threshold current that is DHP sensitive. Both components are sensitive to Cd^{2+}. The same group previously described similar components of Ca current in the native tissue. Quantitative work awaits larger signals.

Ca Channels Can Be Expressed From Smooth Muscle in Oocytes

Both T-type and L-type Ca channels have been described in smooth muscle cells dissociated or in culture (Benham, Hess & Tsien, 1987). While the physiological role of T-type Ca channels remains basically unknown in these preparations, L-type Ca channels are involved directly or indirectly in smooth muscle cells in the development of the contractile response. L-type Ca channel pharmacology is also of great therapeutic interest, since Ca antagonists and particularly dihydropyridines act on smooth muscle cells and are widely used in pathological situations such as hypertension.

Poly(A^+) RNA was extracted from myometrium of pregnant rat (18 days), because electrophysiological studies on such cells in primary cultures revealed optimal expression of Ca channels (Mollard et al., 1986). mRNA-injected oocytes studied in 40 mM Ba express a high threshold Ba current of rather small amplitude (<90 nA), which peaks at potentials around +10 mV and, in contrast with expressed cardiac Ca channels, inactivate with a time constant of ~450 msec at +10 mV. Steady-state half-inactivation occurs at −19 mV (Fournier et al., 1989). These exogenous Ba currents are inhibited partly by 1 μM nifedipine or nitrendipine. Similar electrophysiological properties are found in patch-clamp measurements on single myometrium cells in primary culture and in comparable conditions. High doses of DHP (1 μM) only partially blocked expressed Ca channels, probably because of both (i) the high external Ba concentration (Lory et al., 1990), and (ii) the voltage dependence of the action of these drugs since the effect of DHP was tested at −80 mV. Further work is necessary to compare the pharmacology of ex-

pressed Ca channels from myometrium to those of native cells.

Specific Antisense Oligodeoxyribonucleotides Block Ca Channel Expression

The results described above show, in summary, that Ca channels expressed in oocytes from total RNA or poly(A^+) mRNA have properties that strongly resemble those of native tissue. It can therefore be assumed that all the subunits are assembled to constitute functional Ca channels. On the other hand, tissue-derived RNA induces the expression of several types of ionic channels and probably several subtypes of Ca channels. One way to identify the expression of a given Ca channel type and also to separate two populations of different channels is to inhibit selectively a given RNA expression in *Xenopus* oocytes using antisense oligodeoxyribonucleotides (ODNs). ODNs (15 to 30 nucleotides long) complementary to an mRNA sequence specially inactivate this mRNA in *Xenopus* oocytes probably *via* an RNase H-like degradation mechanism (Dash et al., 1987; Cazenave et al., 1987). Since rat and rabbit heart mRNA express functional DHP-sensitive Ca channel, it was assumed by Lotan et al. (1989) that the major polypeptide had high homology with the skeletal muscle DHP receptor. Since the primary sequence of the DHP receptor from skeletal muscle was known (Tanabe et al., 1987), two ODNs (DHP-01 and DHP-02) were chosen including segments S4 and S3, because membrane-spanning stretches are assumed to be better conserved among different tissues and species. Subsequent cloning of the cardiac channel by Mikami et al. (1989) showed that these regions are in fact quite similar to the skeletal muscle channel. Lotan et al. (1989) used prolonged hybridization (3 to 4 hr) of the heterologous mRNA with the oligonucleotides before the injection in oocytes. Neither DHP-01 or DHP-02 suppressed the expression of voltage-dependent Na and K currents directed from rat or chicken brain. However, both ODNs specifically inhibited DHP-sensitive rat or rabbit heart calcium channel expression. A remaining transient component could reflect the expression of a T-type current. These results confirmed the crucial role in the cardiac L Ca channel protein of a polypeptide with a probable close structural homology with the skeletal $\alpha 1$ subunit.

Diversity of Cloned Ca Channel $\alpha 1$ Subunits

Molecular biological studies have revealed the primary sequences of the $\alpha 1$ subunit of L-type Ca channels from skeletal muscle (Tanabe et al., 1987; Ellis et al., 1988), cardiac muscle (Mikami et al., 1989; Slish et al., 1989), and aortic and pulmonary smooth muscles (Biel et al., 1990; Koch et al., 1990).

Snutch et al. (1990) and independently Perez-Reyes et al. (1990) found a substantial diversity of putative Ca channel $\alpha 1$ subunits in rat brain and hamster endocrine cells by isolating and characterizing several cDNAs that are homologous to the $\alpha 1$ subunit of skeletal muscle. The cDNAs fall into four distinct classes each corresponding to a distinct hybridization pattern of brain mRNA. The classes were termed rbA or 4, rbB or 5, rbC or 2, and rbD or 3, with letters used by Snutch et al. (1990) and numbers by Perez-Reyes et al. (1990); additionally, Perez-Reyes et al. termed the original skeletal muscle $\alpha 1$ subunit class 1. Southern blot and DNA sequencing suggest that each class of cDNA represents a distinct gene or gene family. The class C and D (Hui et al., 1991) gene products share 75 and 71% amino acid identity, respectively, with the $\alpha 1$ subunit of skeletal muscle. The class C sequences show ~95% amino acid identity to the $\alpha 1$ subunit expressed in rabbit lung (Biel et al., 1990) and rat aorta (Koch et al., 1990). On the other hand, class A and B cDNA are less related in primary structure both to DHP-sensitive Ca channels and to class C and D genes. Data from several laboratories now show that $\alpha 1$ subunits from several of these classes are increased in diversity by alternative splicing at the loop between repeats I and II (C: Snutch et al., 1991; Diebold et al., 1991[2]), in the IVS3 regions (C: Biel et al., 1990; Perez-Reyes et al., 1990; Snutch et al., 1991; Diebold et al., 1991[2]; D: Koch et al., 1989; Perez-Reyez et al., 1990; McKenna et al., 1990; Hui et al., 1991), in the IVS3-IVS4 loop (skeletal muscle: Perez-Reyes et al., 1990; C: Perez-Reyes et al., 1990), and in the C-terminal tail (A: Mori et al., 1991).

The functional significance of these brain cDNAs was first assessed using hybrid depletion experiments as described by Lotan et al. (1989). These experiments show that although antisense oligonucleotides against class A and B cDNA partially inhibit, an oligonucleotide against a conserved region of class subtypes almost fully inhibited expression of both brain (Snutch et al., 1990) and heart (Snutch et al., 1991) Ca channels. These results suggest that several classes of Ca channels are expressed in rat brain, although a dominant one with a high homology with the rabbit cardiac Ca channel is related to most of the exogenous Ba current in rat brain mRNA injected oocytes. However, the anti-

[2] Diebold, R.J., Koch, W.J., Ellinor, P.T., Wang, J.J., Muthuchamy, M., Wieczorek, D.F., Schwartz, A. 1991. (*submitted*)

sense suppression results are puzzling in view of the facts that (i) the rat brain mRNA encodes channels whose pharmacology differs from that for the cardiac clone (Mikami et al., 1989) and (ii) full-length A class clones have recently been obtained (Mori et al., 1991; Starr et al., 1991) and produce oocyte currents that resemble these for rat brain mRNA (Leonard et al., 1991; Mori et al., 1991). One should consider nonspecific effects of oligodeoxynucleotide injection at high concentrations (Smith et al., 1990). The issue may be resolved when rbC-I and rbC-II clones are directly expressed in ooxytes. Further analysis of the class A clone expressed in oocytes by Mori et al. (1991) shows that some of its properties (insensitivity to DHP and ω-CgTx, partial inactivation) resemble those of the Ca channels first described by Leonard et al. (1987) after rat brain RNA injection into oocytes. Furthermore, both the class A channels and the rat brain RNA directed channels are sensitive to FTX and were therefore termed "P-type" by Lin et al. (1990). However, there are differences in kinetic and single-channel properties between the class A channels and the "P-type" channels (Tsien, Ellinore & Horne, 1991).

A recent preliminary report (Feldman et al., 1991) suggests that the class D clone encodes L-type channels. Thus a provisional progress report would suggest the following correspondence:

Class A, P type
Class B, unknown
Class C, L type
Class D, L type.

Expression of Cloned Ca Channels is Enhanced by Auxilliary Subunits

The role of $\alpha 1$ as the main, pore-forming, subunit of the calcium channel has been demonstrated by the direct expression of functional calcium channels in *Xenopus* oocytes injected with RNA coding for this subunit only (synthesized in vitro from the correponding DNA). L-type channels from cardiac and smooth muscle (Mikami et al., 1989; Biel et al., 1990) and a non-L type from brain (Mori et al., 1991) were expressed in this way. As noted above, various investigators have found that the skeletal muscle (SkM) calcium channel could not be expressed in oocytes; but transfection of mammalian L cells with cDNA coding for the $\alpha 1$ subunit gives rise to functional L-type Ca channels normally not found in these cells (Perez-Reyes et al., 1989; *see below*).

The role of the other four subunits of the Ca channel (mostly isolated and cloned from skeletal muscle) has been studied recently. In the first reports on this point, co-injection of the SkM α_2/δ with cardiac $\alpha 1$ subunit mRNA in *Xenopus* oocytes improved the expression of the Ca^{2+} channels (Mikami et al., 1989; Biel et al., 1990); and SkM α_2/δ and β subunits also greatly enhanced the expression of a DHP-insensitive brain $\alpha 1$ subunit. The SkM γ subunit seemed to be without significant effect (Mori et al., 1991). However, the kinetic and pharmacological properties of the currents were reported to be unaffected (Mikami et al., 1989, Mori et al., 1991), so it seemed that the major role of the "auxiliary" subunits was to aid in correct assembly of the channel.

In a recent work (Singer et al., 1991), this problem was re-examined by co-expressing the cardiac $\alpha 1$ with the other four auxiliary channel subunits in various combinations in *Xenopus* oocytes, followed by studies on the macroscopic charcteristics of the current through the expressed Ca^{2+} channels. As in the study by Mori et al. (1991), the cDNAs for the auxiliary subunits were from SkM; it must therefore be remembered that the expressed channels were heart/SkM hybrids. Each auxiliary subunit, alone or in combination with the others, had profound effects on biophysical and pharmacological properties of the cardiac Ca^{2+} channel: kinetics of activation and inactivation, voltage dependence, and sensitivity to a dihydropyridine agonist, (−) Bay K 8644.

a) While it is already known that the $\alpha 1$ subunit plays a major role in determining the activation kinetics (Tanabe et al., 1990*a*,*b*; 1991), the $\alpha 2/\delta$ subunit (co-expressed with $\alpha 1$, independently of the presence or absence of β or γ) also strongly influences activation kinetics, bringing the rise time from about 60 msec observed with $\alpha 1$ alone (or other subunit combinations not containing $\alpha 2/\delta$) down to 20 msec, which is close to that observed in mammalian cardiac cells at room temperature. Similar experiments by Mikami et al. (1989) did not however yield this change in kinetics; the discrepancy remains unexplained.

b) The macroscopic voltage dependence of Ca channel activation is similar for channels directed by all subunit combinations containing the cardiac $\alpha 1$, suggesting a major role for the $\alpha 1$ subunit in determining the voltage dependence of the channel. However, other subunits may have a secondary effect (in particular, there is a joint effect of $\alpha 2/\delta$ and β; *see below*).

c) The auxiliary subunits play a major role in determining both the kinetics and voltage dependence of the inactivation process. The Ca^{2+} channel formed by the pore-forming $\alpha 1$ subunit alone shows little time or voltage-dependent inactivation. Addition of any "auxiliary" subunit sharply increases the voltage sensitivity of the inactivation process, with β being the least and γ the most potent; $\alpha 2/\delta$

and γ but not β also strongly accelerate the current decay. If present, γ almost completely dominates the inactivation process, making it faster and more sensitive to voltage. In the absence of γ the inactivation properties vary quite widely depending on subunit composition.

d) The combination of $\alpha 2/\delta$ and β subunits has a synergistic action, very different from the "arithmetic sum" of individual effects of $\alpha 2/\delta$ and β, not only on expression of the channel, but also on channel properties. These include a lower sensitivity to (−) Bay K 8644 than in other subunit combinations, steeper voltage dependence of activation and inactivation processes, and activation at more negative potentials. This suggests a strong interaction between these two subunits (directly or *via* $\alpha 1$).

The dependence of Ca channel properties on the presence of the auxiliary subunits probably does not arise from protection of the channel against proteolysis or enhancement of its insertion into the membrane; instead there seems to be a physical association between the auxiliary subunits and the pore-forming $\alpha 1$ subunit. Furthermore, these data suggest that the interaction between $\alpha 2/\delta$ or γ subunits with the $\alpha 1$ subunit is direct and not *via* another subunit, because each of them alters the properties of the channel when co-expressed with $\alpha 1$ in the absence of the other auxiliary subunits (although interference of some endogenously present $\alpha 2/\delta$, β or γ cannot be completely excluded). The effect of the β subunit alone on the biophysical parameters of the channel is usually negligible (except for a moderate negative shift in the inactivation curve), and its direct contact with $\alpha 1$ is less certain.

Calcium Channel Expression in Mammalian Cells is Also Influenced by Auxiliary Subunits

The rather modest expression obtained in oocytes with the cardiac and smooth muscle $\alpha 1$ subunits alone and the complete failure obtained with skeletal muscle $\alpha 1$ subunits has encouraged investiagtors to attempt expression in mammalian cells. There were two obvious hypotheses. (i) The $\alpha 1$ subunit might need to associate with the $\alpha 2$, β, γ, and/or δ subunits for functional expression. The recent experiments of Mori et al. (1991) and Singer et al. (1991) with oocyte expression support this idea for cardiac and brain Ca channels. Nonetheless, the SkM $\alpha 1$ fails to express in oocytes even when injected with the other SkM subunits. (ii) The SkM $\alpha 1$ subunit might also be processed incorrectly in oocytes. The experiments of Perez-Reyes et al. (1989) tested both of these hypotheses. The SkM $\alpha 1$ subunit was stably transformed into L cells that failed to express any detectable $\alpha 2$ subunit. Modest dihydropyridine binding and calcium currents were obtained, both corresponding to 500–1000 channels per cell. Western blot analysis showed a relative molecular mass (mol wt) of 195 kDa, definitely greater than the apparent mol wt of the T-tubule $\alpha 1$ subunit. Thus, cell-specific proteolytic processing was suggested; processing was later confirmed by the experiments of DeJongh et al. (1990). Furthermore, an obligatory role for the $\alpha 2$ subunit was ruled out. However, expression was still quite low compared to that (>10,000 per cell) recently found in mammalian cells for heterologous expression of Na and K channels; and activation was much slower than normal.

The recent experiments of Varadi et al. (1991) represent the first study in which all cloned SkM subunits ($\alpha 1$, $\alpha 2/\delta$, β, γ) were expressed in various partial and complete combinations in a mammalian cell line (L cells). In this study and in independent studies by Lacerda et al. (1991), much greater expression of DHP binding was obtained by coexpression of $\alpha 1$ and β, although the currents did not increase. Thus, these cells displayed the discrepancy between DHP binding and currents usually observed in skeletal muscle. Furthermore, the β subunit also markedly accelerated both the activation and inactivation kinetics (Lacerda et al., 1991, Varadi et al., 1991).

At this point it is still not clear what additional components, processing steps, or other events are required for robust (>1000 channels per cell) heterologous Ca channel in mammalian cells. The situation may resemble that for Na channel expression: in one study, only cells with endogenous channels were able to express foreign Na channels (Yang et al., 1992).

Expression in Dysgeneic Mouse Muscle

In view of the low current levels in mammalian cells, investigators sought a mammalian system that already expressed the auxiliary subunits and required only an $\alpha 1$ subunit for complete Ca channels. The most likely system would be a skeletal muscle cell, for such cells would presumably process correctly as well. This system is provided by the muscular dysgenesis (MDG) mouse mutant. The stage for these studies was set by the observations that this disease, a fatal autosomal recessive mutation, is expressed in skeletal muscle as a failure of E-C coupling (Powell & Fambrough, 1973; Klaus et al., 1983) and absence of the DHP-sensitive Ca^{2+} current (Beam, Knudson & Powell, 1986; Rieger et al., 1987). This failure appears traceable to a mutation in the structural gene for the SkM $\alpha 1$ subunit (Ta-

Table. Oocytes *versus* mammalian cells for heterologous expression of ion channels

Oocytes	
Advantages	Disadvantages
Cells are very large, allowing simple microinjection of mRNA or DNA and 2-electrode voltage clamp.	Large capacitance leads to poor temporal resolution in voltage-clamp experiments.
Individual cells can easily be identified, manipulated, and retested. A high percentage of cells express the protein.	There are seasonal variations in expression.
Recordings are stable for long periods and extracellular solutions can be simply exchanged.	There are variations between oocytes from different frogs and between oocytes from a single frog.
The cell is quiescent: mRNA injection does not necessarily change the metabolic state.	This is a transient expression system: cells die in a few days.
Intracellular Ca^{2+} is not routinely buffered during experiments; therefore Ca^{2+}-dependent processes are not masked.	The several endogenous conductances may mask expressed channels.
Endogenous conductances may aid in identifying expressed proteins (e.g., Ca^{2+}-activated Cl^- conductance reveals expressed Ca^{2+} channels).	Perfusion for precise control of internal solutions is tedious.
Rapid screening is possible for expression cloning.	

Mammalian Cells	
Advantages	Disadvantages
Cells are well suited for high-resolution patch-clamp recording.	It can be tedious to identify individual expressing cells.
The intracellular solution can be easily controlled by dialysis from a whole-cell patch pipette.	Recordings are stable for tens of min at the most.
There are reliable methods for constructing clonal cell lines that stably express foreign genes.	Intracellular solutions cannot easily be changed.
The large variety of cell lines allows complementation and selection of endogenous channel repertoire.	Each recording requires a new patch pipette, so that screening is inefficient.
High-level expression can be attempted in large-scale cultures.	
Post-translational events (e.g., glycosylation) may be appropriate for expressed mammalian proteins.	

nabe et al., 1988). It thus appeared likely that the MDG mutant would require only a correct $\alpha 1$ subunit for normal function. Tanabe et al. (1988) constructed an expression plasmid driven by the SV40 promoter, including β-globin gene 5′ and 3′ untranslated sequences, and the cDNA for the SkM $\alpha 1$ subunit. This plasmid was microinjected into the nuclei of multinucleated dysgenic myotubes in primary cultures. Forty percent of the myotubes survived the injection; a bit more than 10% of these displayed restored E-C coupling and restored L-type calcium current. Analysis of the injected myotubes with calcium-free external solutions and with external Cd^{2+} indicated that, as in normal muscle, the expressed $\alpha 1$ subunit was re-establishing the normal coupling between depolarization of the surface membrane and release of calcium from the sarcoplasmic reticulum. Thus, the heterologously expressed $\alpha 1$ subunit was functioning primarily both as a voltage sensor and as a calcium channel. The heterologous expression system thus reinforces the concept that skeletal muscle dihydropyridine receptors are vestigial calcium channels—they allow small, slowly activating calcium fluxes that are not necessary for E-C contraction.

Further recent papers from the Tanabe/Beam/Numa group continue to exploit this system. When a cDNA for the cardiac $\alpha 1$ subunit became available, it was inserted into the same expression plasmid and tested in the same system. Again, both E-C coupling and L-type calcium currents were restored to the myotubes; however, the waveforms were appropriate to the source tissue for the $\alpha 1$ subunit. Thus, the cardiac $\alpha 1$ subunit expressed calcium currents that activated within a few msec, whereas those of the skeletal muscle $\alpha 1$ subunit required several hundred msec for activation (Tanabe et al., 1990*b*). The activation was shifted to more negative poten-

tials for the cardiac subunit. Substitution of Ba^{2+} for Ca^{2+} produced a substantial increase in peak current for the cardiac subunit and only a moderate increase for the skeletal muscle subunit. Curiously, however, calcium-dependent inactivation was less prominent for this heterologous reconsititution system than for cardiac tissue.

Of further interest, the cardiac subunit produced E-C coupling that depended on external Ca^{2+}. Finally, effects of repetitive stimulation in caffeine-treated cells showed clearly that Ca^{2+} entry was triggering additional Ca^{2+} release from the sarcoplasmic reticulum. In short, comparison of the skeletal and cardiac $\alpha 1$ subunits expressed in the same cell type produced many of the physiological and pharmacological differences between E-C coupling in the two source tissues.

In the past, site-directed mutagenesis of ion channels has been preceded by a period of experimentation with channels that are chimeras between homologous proteins or, in the case of hetero-oligomeric channels, hybrids in which subunits from different species are systematically exchanged. Tanabe, Beam, Numa and their colleagues have begun such experiments for the $\alpha 1$ subunit as well. Chimeric subunits were constructed containing regions of the skeletal and cardiac muscle $\alpha 1$ sequence, with subsequent analysis of the current waveform, Ca^{2+} dependence of E-C coupling, and intramembrane charged movement ("gating current"). Of the regions investigated, the amino terminal cyctoplasmic tail and the carboxy terminal cytoplasmic tail appeared not to affect the phenotype of E-C coupling. The cytoplasmic loop betweeen homology domains II and III appeared to affect the phenotype of coupling most strongly; contraction became independent of external Ca^{2+}. Substitution of the skeletal muscle I-II loop had a similar but less pronounced effect. Thus, of the four regions studied, the II-III loop seems to be the best candidate for the mechanical link between the membrane potential and Ca^{2+} release from the sarcoplasmic reticulum (Tanabe et al., 1990*a*). Further study of this and other regions is clearly of interest. For all the chimeras, current waveforms were rapid activating, appropriate to the cardiac $\alpha 1$ subunit.

In charge movement studies (Adams et al., 1990), significant technical effort was required to eliminate the gating currents associated with Na^+ and fast Ca^{2+} channels; there may still have been contamination by K^+ channel gating currents. The major parameter studied was the ratio of Ca^{2+} conductance to charge movement; this parameter should normalize data obtained at varying expression levels. The data showed a curious correlation between the phenotype of E-C coupling and that of charge movement. The chimera with skeletal type E-C coupling showed the highest ratio of calcium conductance to charge movement, even though the skeletal muscle $\alpha 1$ subunit is thought to retain only vestigial conductance properties. Perhaps the most significant result of this study is the confirmation that a DHP-sensitive Ca channel is indeed responsible for the bulk of intramembrane charge movement in normal skeletal muscle. The results also lend credence to the hypothesis that the S4 region, which is similar between the two $\alpha 1$ subunits, senses voltage by moving within the membrane field and thus produces the intramembrane charge movements. In the author's words, "participation in direct, skeletal type E-C coupling does not necessarily cause a DHP receptor to lose its efficiency as a calcium channel."

In more recent experiments on 15 different chimeric skeletal/cardiac muscle $\alpha 1$ subunit chimeras, Tanabe et al. (1991) found a clear correlation between the presence of the homology repeat I and Ba currents that display (i) rapid activation upon depolarization and (ii) rapidly deactivating tail currents upon repolarization. It therefore appears that repeat I plays a special function role in voltage-dependent gating; perhaps the conformational transitions in this repeat are the slowest, rate-limiting steps.

Concluding Remarks

The voltage-dependent Ca channel family provides an interesting range of pharmacology, kinetics, modulations, and permeation properties that well suit the wide range of physiological roles for these channels. This rich variety far exceeds the spectrum of Na channels and may match the variety of K channels. Yet the molecular biology and heterologous expression of Ca^{2+} channels has lagged significantly behind similar experiments for Na^+ and K^+ channels. With the recent description of multiple channel clones from rat brain and the recent demonstration that oocytes can indeed express several Ca channel types at robust levels if supplied with RNA for the appropriate auxiliary subunits, the picture has changed completely. We expect significant advances in knowledge about (i) structure-function relations, (ii) sorting and targeting to particular cell regions, (iii) interactions with G proteins, and (iv) drug binding sites. We are personally delighted with the opportunity to participate in some of these studies.

We thank A. Schwartz for comments on the manuscript. Work in our laboratories on these topics and preparation of this review was supported by the Centre National de la Recherche Scientifique, the Institut National pour la Recherche Médicale, the Asso-

ciation Française contre les Myopathies, NATO (CRG 890374), the U.S.-Israel Binational Science Foundation (90-00053), and the National Institutes of Health (GM-29836).

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Received 12 July 1991; revised 7 November 1991