Gerald Werner Zamponi
Norbert Weiss *Editors*

Voltage-Gated Calcium Channels

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ISBN 978-3-031-08880-3 ISBN 978-3-031-08881-0 (eBook)
https://doi.org/10.1007/978-3-031-08881-0

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

In 2004, we were asked whether we would be willing to organize a book on inactivation mechanisms in calcium channels. This seemed overly focused, and we made a counterproposal to have a comprehensive book on calcium channels. It took almost 18 months to collect the more than 20 chapters, but the end result was a comprehensive book that covered the state of knowledge on calcium channels at the time.

Much has happened globally since 2004. The world is ruled by social media, and Facebook, YouTube, Twitter, and Instagram are now part and parcel of our science communications, but they have also served as platforms for science denial and disinformation. The publishing world has been transformed through rapid online publications, but it has been inundated with a plethora of predatory journals that pollute the purity of scientific output. Through these changes, the calcium channel field has endured, but there have been a number of changes.

Prior to the early 2000s, large scientific conferences, such as the Society for Neuroscience annual conference or the Biophysical Society meetings, had multiple oral sessions dedicated to calcium channels, and this is now no longer the case. As a way of bringing the calcium channel field together, we organized five international calcium channel conferences, always in a different location across the globe. This was subsequently complemented by the European calcium channel meeting organized by colleagues at the University of Innsbruck. These types of activities have fostered scientific exchange and helped build a sense of camaraderie, and many of the regulars at these conferences are participants in this book. It is interesting to note that a dozen of the authors of the current book also contributed to the earlier book, in some cases as trainees who are now firmly established leaders in the field, and there are many contributors who constitute a newer cadre of calcium channel experts. Sadly, we lost one of the true greats of calcium channel physiology due to the unfortunate passing of Dr. David Yue in 2014, but his legacy lives on through the outstanding work by his former trainees such as Drs. Dick and Ben Johny.

There have been numerous advances in the calcium channel field over the past two decades. Due to rapid genetic sequencing, the field of calcium channelopathies has expanded dramatically, and has given rise to parent-led organizations such as the CACNA1A foundation. The availability of new technical approaches such as CRISPR has helped provide deeper insights into the physiological and pathophysiological roles of calcium channels. Perhaps, most impressively, the availability of crystal and cryo-EM structural informa-

tion as highlighted by Dr. Catterall has dramatically advanced the field and will have a major impact on drug discovery for the treatment of calcium channel–related disorders.

The 2022 edition of *Voltage-Gated Calcium Channels* builds on the large body of information contained in the earlier book, but greatly extends this content to incorporate these new and exciting developments. The first section covers the most important structural and molecular aspects of voltage-gated calcium channels and their associated ancillary subunits, but also the importance of splice variation and RNA editing which contribute to extend their diversity and physiological roles. The second section deals with the different aspects of their regulation, ranging from their genetic control to their trafficking and regulation at the plasma membrane, and also their association with other channels and receptors. The third section is dedicated to their implication in the development of human disorders, a field that has expanded dramatically over the last years and continues to be one of the fastest growing research areas. Finally, the fourth section naturally covers the different aspects of their pharmacology, ranging from molecular pharmacology to a variety of small molecules and peptides that not only represent invaluable tools for the physiological study of calcium channels but also represent potential pharmacological tools for therapeutic intervention.

We would like to warmly thank all of the authors without whom this second edition would not have been possible. We believe that to have all of this information in a single volume provides a fantastic resource for both those actively involved in the field and for those wishing to find out more about particular aspects of calcium channels.

Calgary, AB, Canada — Gerald Werner Zamponi
Prague, Czech Republic — Norbert Weiss

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Part III (Patho)physiology of VGCCs

A Lived History of Early Calcium Channel Discoveries Over the Past Half-Century

Emilio Carbone

Abstract

This chapter of the book is directed to PhD students, post-docs and young researchers who are attracted by the unique properties of voltage-gated calcium channels. This chapter aims to provide an overview of the most important discoveries that helped elucidate the structure and function of calcium channels in excitable cells. While systematically reviewing the numerous works in the field, I chose to write a personal story derived from my own experience on Ca^{2+} channels, as it developed in the lab and through the discussions with many colleagues working on ion channels. This occurred in a period in which Ca^{2+} channels reached maximal attention among scientists and brought the many astonishing achievements described in this book. Given the broad interdisciplinarity of Ca^{2+} channel discoveries, the present history may probably appear incomplete, but certainly, the other chapters of this book will cover all possible gaps on the matter.

Keywords

Ca-spikes · Ca^{2+} currents · L-type · N-type · P/Q-type · R-type · T-type channels · Calcium channels structure and function · GPCR-mediated inhibition of Cav2 channels

Ca^{2+} as Central Ion for Muscle Contraction

The relevance of calcium ions in myocardial function is about 140 years old. It coincides with Sydney Ringer's observations on the ionic constituents of blood on heart contraction published in the newly founded *Journal of Physiology* (Ringer, 1883). By changing the ionic composition of bath solutions used to keep frog ventricles contracting, Ringer discovered that Ca^{2+} is the key extracellular cation required to maintain the regular heart beating (see Fye, 1984 for curious aspects of Ringer's experiments). Ringer's observation was soon extended to smooth muscle contraction (Stiles, 1901), and 60 years later, T. Kamada (Japan) and L.V. Heilbrunn (USA) uncovered the role of Ca^{2+} on skeletal muscle contraction (Kamada & Kinoshita, 1943; Heilbrunn & Wiercinski, 1947). Following this, calcium was progressively identified as a determinant constituent of extracellular solutions in all excitable cells and recognized as the most

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G. W. Zamponi, N. Weiss (eds.), *Voltage-Gated Calcium Channels*,
https://doi.org/10.1007/978-3-031-08881-0_1

widespread and versatile signalling element involved in many cellular processes (Carafoli et al., 2001). Intracellular Ca^{2+} is now considered the most ubiquitous second messenger capable of modulating numerous cell functions (Berridge et al., 2003). Initiation of intracellular Ca^{2+} signalling events obviously requires Ca^{2+} movement across the plasma membrane, and this, in the majority of cells, involves the presence of Ca^{2+} permeable channels.

The "Ca-Spikes" of Crustacean Skeletal Muscles

Following the four revolutionary papers by Alan Hodgkin and Andrew Huxley on the ionic basis of action potential (AP) generation in the squid giant axon (Hodgkin & Huxley, 1952a, b, c, d), it soon became evident that Na^+ currents alone were not sufficient to account for the generation of the AP upstroke in several excitable cells. Thanks to the strong driving force for Ca^{2+} at the threshold potential where APs originate, other ions, like Ca^{2+}, could potentially carry enough inward current to sustain the APs. Paul Fatt, Bernard Katz and Bernard Ginsborg were the first to recognize the importance of transmembrane Ca^{2+} fluxes to sustain APs in crustacean muscle fibres (Fatt & Katz, 1953) and to propose that a Ca^{2+} current was responsible for the APs in the absence of external Na^+ (Fatt & Ginsborg, 1958). Similar findings on Ca-dependent APs were reported in the following years in other crustacean muscle fibres using variable Na^+/Ca^{2+} concentration ratios in the external solution to test for the amplitude and shape of APs (for a review see Reuter, 1973). However, the most direct information regarding the ability of Ca^{2+} ions to serve as charge carriers during excitation in skeletal muscle came from the studies of Susumu Hagiwara (USA) on barnacle muscle fibres. Hagiwara's group showed that in the absence of external Ca^{2+} the APs stopped, regardless of the external $[Na^+]$ concentration. The overshoot of the spike increased with increasing extracellular $[Ca^{2+}]$ (Fig. 1a), and all-or-none spikes could be recorded when the intracellular $[Ca^{2+}]$ was reduced by injecting various Ca^{2+}-binding agents (Hagiwara & Naka, 1964). Using TTX and local anaesthetics, Hagiwara and colleagues could clearly separate "Na-spikes" from "Ca-spikes" and identify manganese (Mn^{2+}) as an effective blocker of Ca^{2+} influx (Hagiwara & Nakajima, 1966).

"Ca-Spikes" in Heart and Neurons

The existence of a Na^+ and Ca^{2+} inward current system that regulates AP waveforms and their amplitudes became soon evident also in cardiac tissues and mollusc neurons. In the frog heart, for instance, it became apparent that the rising phase of the AP was composed of two phases: one fast, Na^+ driven and one slower, Ca^{2+} driven (Wright & Ogata, 1961; Niedergerke & Orkand, 1966). The same was observed in mammalians heart cells (Stanley & Reiter, 1965; Paes de Carvalho et al., 1966), confirming the existence of Ca-spikes in heart muscle fibres.

In parallel to skeletal muscle and heart cells, Ca-spikes were described also in several nerve preparations. Initial observations were made on frog spinal ganglion cells (Koketsu et al., 1959; Nishi et al., 1965) and pulmonate mollusc neurons (Oomura et al., 1961). In the first case, frog ganglion cells were shown to produce prolonged APs in Na^+-free solutions when quaternary ammonium ions were present. Ca-spikes were abolished when Ca^{2+} was withdrawn from the Na^+-free solution and were preserved when Ba^{2+} replaced Ca^{2+}. The AP overshoot increased in Na^+-free solutions in the presence of Ba^{2+}, suggesting that Ca^{2+} (or Ba^{2+}) carries charges across the membrane of spinal ganglion cells. In the second case, the giant nerve cells of pulmonate mollusc were shown to produce all-or-none APs in Na^+-free solutions. APs were abolished by removing Ca^{2+} from the solution. Following this, "Ca-spikes" became popular and were reported in many other nerve cell types (Gerasimov, 1965; Geduldig & Junge, 1968; Koketsu & Nishi, 1969).

It is also worth mentioning that Ca^{2+} fluxes were recorded not only at the soma but also at the axonal level. TTX-insensitive

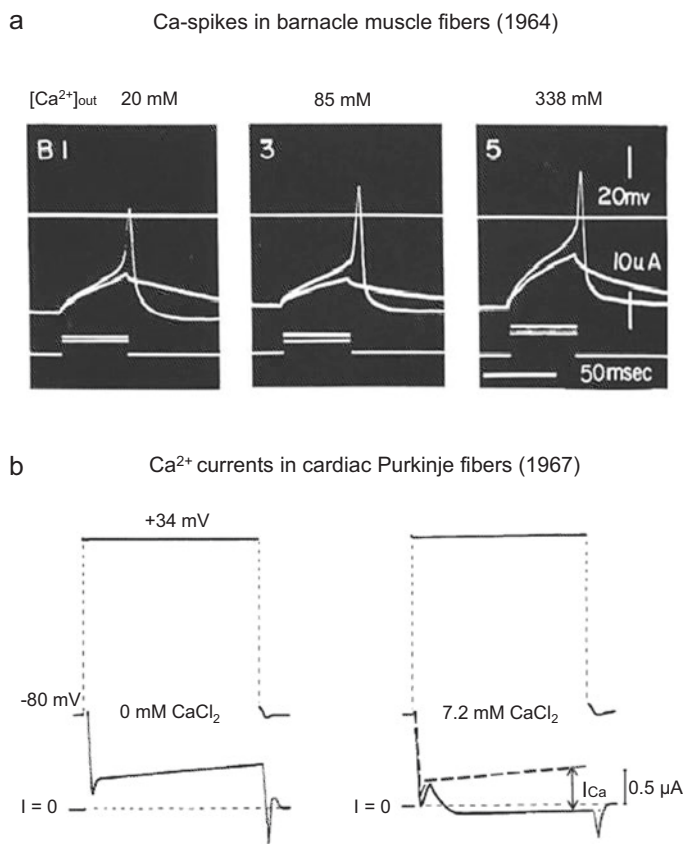

Fig. 1 "Ca-spikes" and Ca^{2+} currents in muscle fibres (1964–1967). (**a**) Effects of external Ca^{2+} on Ca^{2+}-AP overshoot on barnacle muscle fibres. The muscle fibre was injected with K^+_containing solutions. $[Ca^{2+}]_{out}$ is indicated on top of each panel. The top straight line indicates the 0 mV potential. (Redrawn with permission from Hagiwara & Naka, 1964). (**b**) Bottom, Ca^{2+} current recorded from a sheep Purkinje fibre bathed in Na^+-free solution containing 0 mM (left) or 7.2 mM (right) $CaCl_2$, at +34 mV from Vh = −80 mV. The net inward Ca^{2+} current on the right panel (ICa) was calculated by subtracting the current trace of the left panel (dashed curve). (Redrawn with permission from Reuter, 1967)

Ca^{2+} fluxes in the squid giant axon were too small to generate Ca-spikes. Nevertheless, they could be detected through the light produced by the Ca^{2+}-sensitive bioluminescent protein *aequorin* injected in the axon (Baker et al., 1971; Hallett & Carbone, 1972). Ca^{2+} entry during depolarizing voltage pulses was divided into an early TTX-sensitive component, plus a late TTX-insensitive component. Mn^{2+}, Co^{2+}, La^{3+} and organic Ca^{2+} antagonists (D-600 and iproveratril) effectively blocked this component (Baker et al., 1973). At variance with the squid axon, TTX-insensitive Ca-spikes were successfully recorded in *Aplysia* axons in Na^+-free TTX-containing solutions (Horn, 1978). Under these conditions, Ca^{2+} currents were the only currents responsible for propagating APs. The overshoot and maximum rate of rise of Ca-spikes increased with increasing external Ca^{2+}. Co^{2+} or Cd^{2+} blocked these currents and Sr^{2+} or Ba^{2+} could substitute for Ca^{2+} to sustain the AP. In conclusion, axons and cell bodies were able to generate and propagate Ca-spikes (see Hagiwara & Byerly, 1981 for a review).

How to Look at Ca^{2+} Currents Through Voltage-Clamp Recordings

Together with recording and characterizing Ca-spikes in a multitude of excitable cells, the increased availability and popularity of the "voltage-clamp" technique helped resolving the key properties of voltage-gated Ca^{2+} currents in excitable cells. As shown by Hodgkin and Huxley (1952a, b, c, d) for the Na^+ and K^+ conductances of squid axon, the "voltage-clamp" technique allows measuring the voltage-dependent kinetics of activation, inactivation and closing of active ion conductances.

Voltage-clamp recordings have great advantages with respect to current-clamp recordings but have strong limitations concerning the cell shape. Cells need to be either spherical (cell bodies without dendrites) or cylindrical (axons with no branches) and, most importantly, should have large dimensions (>150 μm diam) to allow the positioning of "two electrodes", one for voltage command and one for current recordings. The squid giant axon is ideal for voltage-clamp recordings. It has a quite homogeneous cylindrical shape of large diameter (300–800 μm), so that by using low-resistance platinum axial current electrodes and thin axial voltage glass microelectrodes, it is possible to record Na^+ and K^+ currents with high-time resolution (Armstrong, 1966, 1969). These conditions were quite limiting for recording Ca^{2+} currents from muscle and neuronal preparations that exhibited Ca-spikes. Therefore, the first critical issue to solve was to find proper cell preparations and voltage-clamp approaches to measure Ca^{2+} currents at fixed potentials.

Ca^{2+} Currents in the Heart and Mollusc Neurons: The Problem of Blocking K^+ Currents

In 1967, Harald Reuter (Switzerland) succeeded to measure Ca^{2+} currents in cardiac Purkinje fibres bathed in Na^+-free solution (Reuter, 1967), using the double sucrose voltage-clamp technique developed by Josef Dudel and Wolfang Trautwein in Homburg (Germany) (Dudel et al., 1966). Reuter's experiments gave clear indications that cardiac Ca^{2+} currents were robust and had a slower time course with respect to Na^+ currents. Their identification was not trivial. Ca^{2+} currents in 7.2 mM Ca^{2+} were separated from the dominant outward currents recorded in 0 mM Ca^{2+} by subtraction (Fig. 1b). Interestingly, the isolated Ca^{2+} currents activated with sigmoidal voltage dependence between −60 and +10 mV and zeroed at ~ +150 mV, near the Ca^{2+} equilibrium potential. Since then, the "slow Ca^{2+} currents" were resolved and described in many other heart cell preparations (see Reuter, 1973; Trautwein, 1973 for a review).

The voltage-clamp technique was also successfully applied to measure the Ca^{2+} inward currents responsible for the Ca-spikes of mollusc neurons (Geduldig & Gruener, 1970; Krishtal & Magura, 1970; Kostyuk et al., 1974), including autorhythmic Helix neurons (Eckert & Lux, 1975). In parallel to this, Ca^{2+} currents were characterized in many different cells, establishing the importance of Ca^{2+} channels as molecules coupling cell excitation with intracellular Ca^{2+} signalling (see Hagiwara & Byerly, 1981 for a review).

Most of these works reported the existence of Ca^{2+} currents with rather similar time course and voltage-dependent activation. The currents reached a peak within few milliseconds at about +10 mV and then inactivated with variable time courses. However, the current decay after the peak (inactivation) depended strongly on the block of outward K^+ currents, which were prominent in these neurons and incompletely blocked due to the difficulty to replace intracellular K^+ with commonly used K^+ channel blockers (Cs^+, TEA^+, TMA^+). In spite of this, Paul Brehm and Roger Eckert (USA) could prove that Ca^{2+} current inactivation was effectively regulated by Ca^{2+} fluxes in *Paramecium* (Brehm & Eckert, 1978) and that this feedback signal was fundamental to autoregulate Ca^{2+} entry during cell stimulation to preserve low cytoplasmic Ca^{2+} levels. Eckert and his colleagues invented the "double-pulse" protocol that we still use routinely nowadays to define the degree of

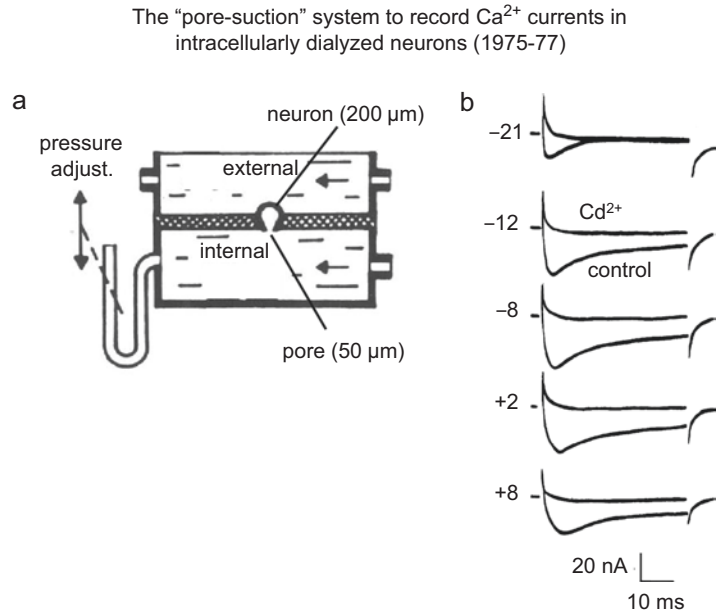

Fig. 2 The "pore-suction technique" to record Ca^{2+} currents in intracellularly dialysed mollusc neurons (1975–1977). (**a**) Experimental set-up for the intracellular dialysis of isolated neurons. The neuron is positioned in the conical hole of the partition. A normal negative pressure in the internal compartment stabilizes the neuron in the hole, while a strong transient negative suction breaks the membrane and creates a pore on the internal side of the neuron. Following this starts the intracellular dialyses and ion current recordings. (**b**) Ca^{2+} currents in a *Helix* neuron dialysed with Tris-phosphate and kept in Na-free solution (Ca^{2+} 10 mM) before (control) and after adding 2 mM Cd^{2+}. (Redrawn with permission from Kostyuk et al., 1977)

Ca^{2+}-dependent inactivation (CDI) of the different Ca^{2+} channels expressed in various excitable cells (see Eckert & Chad, 1984 for a review).

The problem of K^+ current contamination was solved by Platon Kostyuk and Oleg Krishtal in Kiev (Ukraine) by developing an intracellular perfusion method on mollusc (Kostyuk et al., 1975) and mammalian neurons (Krishtal & Pidoplichko, 1980). Kostyuk and Krishtal invented an ingenious system in which a neuron was held firmly into a conical pore within a partition separating the external from the internal solution (Fig. 2a). A transiently applied suction from the intracellular compartment caused the rupture of the cell membrane and complete replacement of the cytoplasm with a solution containing Ca^{2+} chelators and Cs^+ to block K^+ channels (see Kostyuk et al., 1981). Complete block of voltage-gated and Ca^{2+}-activated K^+ currents in the presence of TTX allowed for the first time to record Ca^{2+} currents in isolation, displaying the real voltage-dependent characteristics of neuronal Ca^{2+} channels uncontaminated by K^+ outward currents (Fig. 2b). Modifications of this method were soon proposed using large glass pipettes to hold cells of 150–250 µm diameter. In one case, a negative pressure was created on marine eggs (Takahashi & Yoshii, 1978) while, in another case, mollusc neurons were punched with a metallic wire (Lee et al., 1978) to break the membrane at one side and get access to the intracellular compartment.

A Convergent View on the Existence of "a" Ca^{2+} Channel in Excitable Cells

The works on mollusc neurons and the many others following in that period converged on a commonly accepted view that physiologically and pharmacologically isolated Ca^{2+} currents reflected the time course of Ca^{2+} fluxes through specific cell membrane pathways generically indicated as "Ca^{2+} channels" (see Reuter, 1979;

Kostyuk, 1980; Hagiwara & Byerly, 1981). Ca^{2+} current recordings made it possible to look closer at the role of these channels in the regulation of heart beat, muscle contraction, neuronal excitability and cell exocytosis, and also revealed modulatory effects of hormone and neurotransmitters on these channels (Tsien et al., 1972; Dunlap & Fischbach, 1978).

At this time, there was a general agreement among all groups that nearly all excitable cells expressed only "one type" of Ca^{2+} permeable channel with well-defined characteristics. The channel activated in a voltage-dependent manner at potentials more positive than Na^+ channels and had slower activation kinetics. Inactivation was also slower but, differently from Na^+ channels, was voltage and Ca^{2+} dependent. All these properties fit nicely with the idea that the Ca^{2+} channel was responsible for the broadening of APs in nearly all excitable cells. Broad APs are fundamentals to allow sustained Ca^{2+} entry during heart contraction, neurotransmitter secretion and neuronal signalling with respect to the narrow spikes of the squid axon that serve mainly to conduct excitability along the axonal cable. It was also well proved that Ca^{2+} channels were rather selective for divalent vs. monovalent cations and that Ba^{2+} and Sr^{2+} were more permeable than Ca^{2+} through the open pore (Hagiwara & Byerly, 1983). For more details on the permeability properties of Ca^{2+} channels, please see the review by (Sather and McCleskey, 2003).

The "Patch-Clamp" Technique and the Explosive Interest on Ca^{2+} Channels

All the above discoveries on isolated Ca^{2+} currents were rapidly overwhelmed by the advent of the "patch-clamp" technique that Erwin Neher and Bert Sakmann (Germany) developed to record single ACh receptor channels from "electrically isolated" membrane patches of skeletal muscles (Neher & Sakmann, 1976). Within the next few years, the technique was further refined by introducing a "negative-suction system" to form high-resistance seals (giga-seals) between the glass pipette and the cell membrane that enabled higher current resolution, physical isolation of membrane patches and direct recording of whole-cell and single-channel currents from cells of a small diameter (<20 μm) (Hamill et al., 1981).

The patch-clamp technique boosted in an impressive way the interest on single Ca^{2+} channels and whole-cell Ca^{2+} currents in a variety of vertebrate and invertebrate cells. Snail neurons (Lux & Nagy, 1981), chick DRG neurons and PC12 cells (Brown et al., 1982), bovine chromaffin cells (Fenwick et al., 1982), rat and guinea pig ventricular myocytes (Reuter et al., 1982; Cavalié et al., 1983) and rat pituitary cells (Hagiwara & Ohmori, 1983) were the first preparations used to record single channel and macroscopic Ca^{2+} currents. Thanks to the "patch-clamp" technique, the interest moved quickly from the properties of Ca^{2+} channels in large-size mollusc and marine animal cells to small-size vertebrate cells. I also moved in March 1983 from the lab of Franco Conti and Enzo Wanke at the Institute of Cybernetics and Biophysics in Camogli (Italy), where I was working on Na^+ and K^+ channels of squid axons (Wanke et al., 1980; Carbone et al., 1982), to Hans Dieter Lux' lab of Neurophysiology at the Max Planck Institute for Psychiatry, in Martinsried (Germany).

At that time (1983–1985), Lux's and other groups were interested in the many unsolved problems related to the kinetics, permeability and modulatory properties of Ca^{2+} channels, as well as to the existence of new Ca^{2+} channel types that could be identified biophysically. The accepted paradigm was that probably only one Ca^{2+} channel existed, although the first evidence for a dual Ca^{2+} channel population (types I and II) was obtained already in 1975 using a two-electrode voltage-clamp method in starfish eggs (Hagiwara et al., 1975). Hagiwara's group could show that starfish eggs displayed two channel types: one activating at relatively negative potentials (−50 mV; type I) and one at more positive voltages (−7 mV, type II). Type I inactivated relatively fast with respect to type II, which closely resembled the "slow Ca^{2+} current" of cardiac cells and mollusc neurons. Type I channel caught

immediately the attention since it activated at low voltages, inactivated rapidly and inactivated steadily following conditioning pre-pulses to −35 mV, similar to Na^+ channels. Surprisingly the time course and amplitude of type I current was found sensitive to the external Na^+ concentration. External Na^+ slowed-down markedly the inactivation of type I channel, possibly due to an unexplained effect of Na^+ on channel gating and permeation, unusual for a vertebrate Ca^{2+} channel.

The Discovery of the "Low-Voltage Activated" T-Type Channel

Evidence for two types of voltage-gated Ca^{2+} channels became soon evident while patching cultured chick sensory neurons under ionic conditions in which only pharmacologically isolated Ca^{2+} currents were recorded, using TTX and high $[Ca^{2+}]$ in the bath and high $[Cs^+]$ and Ca^{2+} chelators in the pipette (Carbone & Lux, 1984b). In nearly every neuron, a "low-voltage activated" (LVA) component emerged transiently at very negative potentials (~ −50 mV) with fast activation and complete inactivation, while a "high-voltage-activated" (HVA) component activated at potentials positive to −20 mV (Fig. 3a). The HVA current was fast activating and slowly inactivating. This current closely resembled the Ca^{2+} current described in other neurons and was therefore less of a focus in these experiments. LVA currents increased with Ca^{2+} (Fig. 3b) and occasionally could be recorded in isolation from HVA currents (Fig. 3c) (Carbone & Lux, 1984a).

More convincing evidence for the presence of a "low-voltage-activating and fully inactivating" Ca^{2+} channel came in parallel while recordings single Ca^{2+} channels in membrane-excised patches. Using the outside-out configuration that allows better control of transmembrane voltage and replacement of internal K^+ ions with Cs^+, Lux and I uncovered the fast bursting activity of single (or multiple) LVA Ca^{2+} channels (Carbone & Lux, 1984a). The channel activity was strictly holding potential (V_h) dependent. With V_h near resting potentials (−60 mV), the channel was silent during depolarizations to −40 mV but was very active after holding the patch to −100 mV (Fig. 4a, left). Repeated openings started soon after the patch depolarization and terminated at increasingly shorter times with increasing voltages. By summing up a sufficient number of single-channel traces at a fix potential, it was evident that the channel fully inactivated during pulses of 200 ms at −20 mV (Fig. 4a, right), mimicking the time course of macroscopic LVA currents.

Lux and I were lucky that the LVA channel resisted to the outside-out patch dialysis so that we could record the activity of the channel for the time required to accumulate a sufficient number of traces for the analysis. We were on the contrary not careful enough in selecting the permeant ion to better separate LVA from HVA channels. Ba^{2+} would have been certainly a better choice to separate biophysically the two channels since Ba^{2+} is more permeable than Ca^{2+} through HVA but not through LVA channels (Fedulova et al., 1985) and would have made the separation during single channel recording easier. We preferred to use 20 mM Ca^{2+} simply to increase the size of the unitary events without excessively altering the external divalent cations concentration. In conclusion, we erroneously estimated the conductance of the LVA fully inactivating channel at twice as much of the real value (Carbone & Lux, 1987a), possibly due to the dual overlapping of more than one active channel in the outside-out patches. However, the permeability to Ca^{2+}, the low voltage-dependent activation, the fast and full inactivation, the sensitivity to the holding potential and the persistent activity in excised patches were the exact fingerprints of the expected single LVA channel, as we know today.

One year later, Dick Tsien's group (USA) found that in cell-attached patches, 110 mM Ba^{2+} and BayK8644 could better separate LVA and HVA channels in chick sensory neurons (Nowycky et al., 1985) and guinea pig ventricular cells (Nilius et al., 1985). Tsien's group brought also evidence for a third type of voltage-gated Ca^{2+} channel in neurons. The three channels were termed: T-type for transient (LVA), N-type for "neither T nor L" (HVA) and L-type for

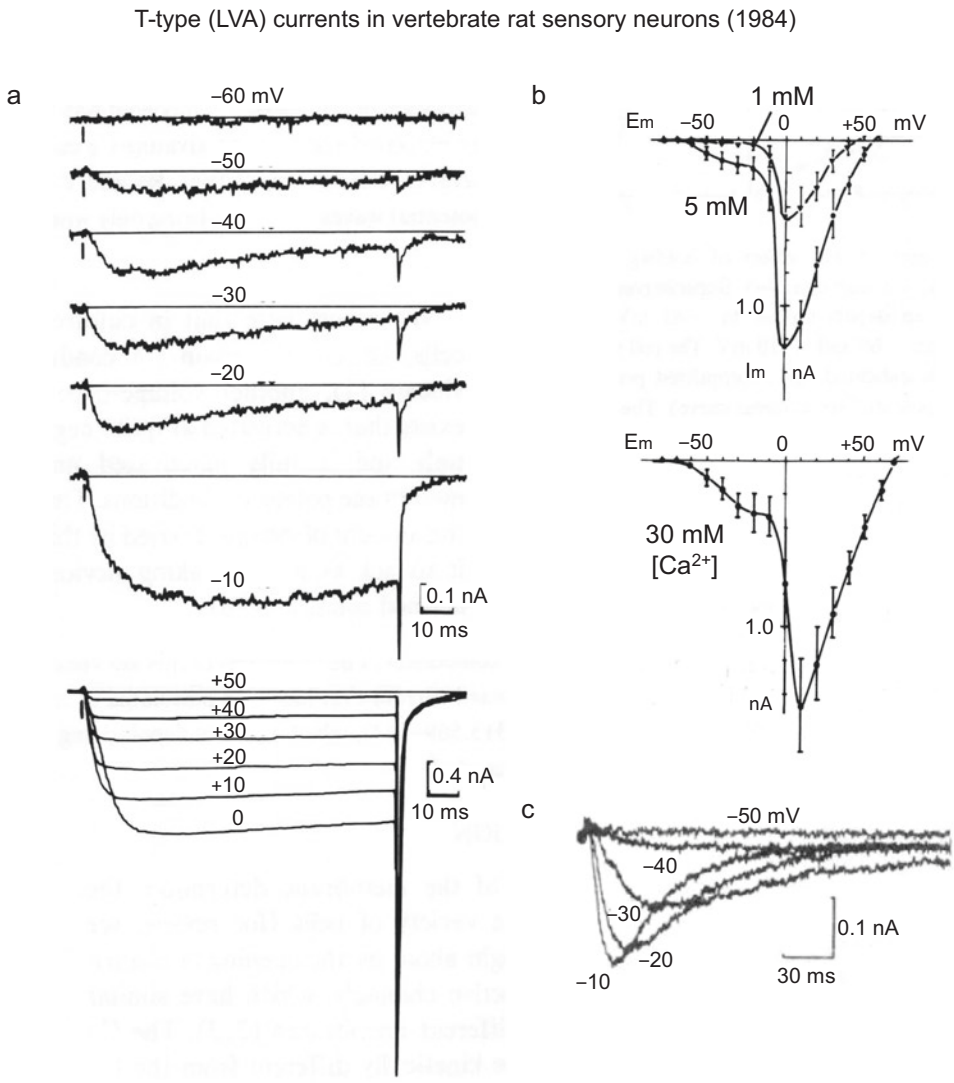

Fig. 3 Whole-cell LVA (T-type) Ca^{2+} currents in chick and rat DRG neurons (1984). (**a**) LVA and HVA whole-cell clamp Ca^{2+} currents recorded from a chick sensory neuron bathed in 5 mM Ca^{2+} plus 3 μM TTX at the potential indicated (Vh −80 mV; 130 mM CsCl in the pipette). (**b**) I-V relationships in 1, 5 and 30 mM Ca^{2+} in chick DRGs. (Redrawn with permission from Carbone & Lux, 1984b). (**c**) LVA (T-type) Ca^{2+} currents recorded in isolation from a rat DRG neuron at the potential indicated (Vh −90 mV). (Redrawn with permission from Carbone & Lux, 1984a)

"long-lasting" (HVA). This terminology became soon popular and often used to indicate the existence of these three channels in a variety of cell preparations. Even nowadays, after the Ca^{2+} channel nomenclature has been drastically modified to account for the newly uncovered Ca^{2+} channel types (Ertel et al., 2000), the T-, N- and L-type terminology is still commonly used.

The Unique Properties of T-Type (LVA) Channels

Concurrently, it became evident that besides activating transiently at very negative membrane potential the neuronal T-type (LVA) channel possessed several other unique properties. Two of them concern the very slow deactivation rate

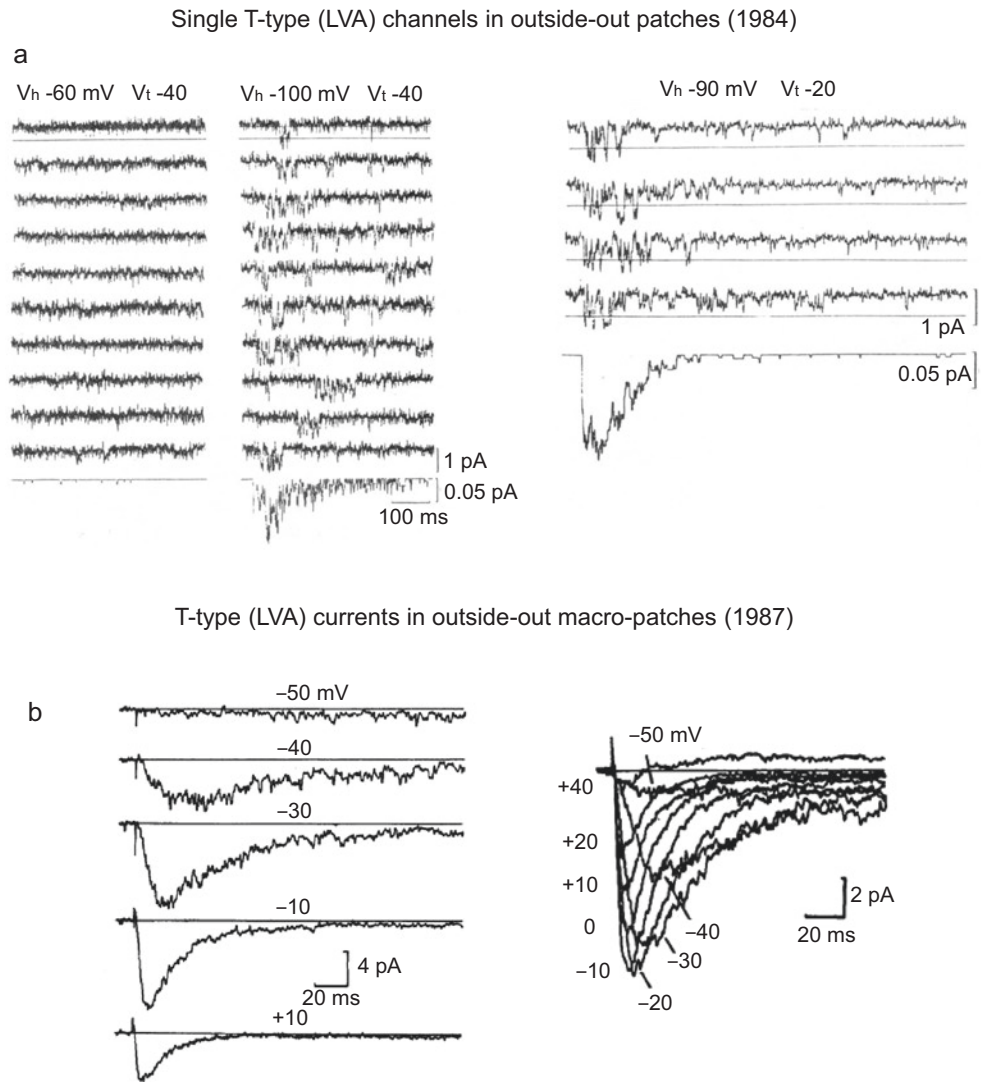

Fig. 4 Single LVA (T-type) channels in outside-out patches and macro-patches of sensory neurons. (**a**) Left, unitary Ca^{2+} currents recorded from an outside-patch of chick DRG neuron bathed in 20 mM $CaCl_2$, 120 mM Choline-Cl and 3 μM TTX (100 mM CsCl and 16 mM TEACl in the pipette) during sequential depolarizations to −40 mV (Vt). Average currents are shown at the bottom. T-type channel activity is visible only when the holding potential (Vh) is lowered to −100 mV (right panel). Right, transient activity of T-type channels recorded during sequential depolarizations to −20 mV. The averaged current (*bottom trace*) is fully inactivated after 200 ms. (Redrawn with permission from Carbone & Lux, 1984a). (**b**) Left, T-type Ca^{2+} currents recorded in an outside-out macro-patch from a rat DRG neuron in 20 mM Ca^{2+} at the potentials indicated. Right, superimposed average traces recorded as in the left panel. Each trace is the average of five records (Vh −80 mV). Apparently, the outside-out macro-patch of rat DRG neurons contains only active T-type channels. (Redrawn with permission from Carbone & Lux, 1987a)

(Carbone & Lux, 1984b; Armstrong & Matteson, 1985) and the effective recruitment of full size low-threshold Ca^{2+} currents following short hyperpolarization (Carbone & Lux, 1987b; Crunelli et al., 1989). These features could nicely account for the existence of Ca^{2+}-mediated

rebound spikes observed in the inferior olive and thalamic neurons (Llinas & Yarom, 1981; Llinas & Jahnsen, 1982), named "low-threshold Ca^{2+} spikes" (for a review see Dreyfus et al., 2010). It is curious how Lux and I became aware of Rodolfo Llinas' papers on the existence of a "low-threshold" Ca^{2+} channel conductance in olivary and thalamic neurons (1981–1982) while writing our manuscripts. We were discussing nearly every day with the lab people about a possible function for the new neuronal LVA channel. Unexpectedly, one day Arthur Konnerth (Germany), at that time post-doc in Lux's lab, came to my desk and very excitedly showed me the two papers by Llinas's group. Unforgettable was the excitation we had with Lux and Arthur while reading the two papers. We were among the first quoting these two excellent works.

Another important feature of the T-type channel regards its high resistance to run-down (loss of activity) following patch excision or dialysis of the cytosol. T-type channels are more resistant to run-down than any other HVA channel (L and N). This allowed, for instance, to easily record T-type channels in isolation by simply using macroscopic outside-out patches (Fig. 4b, left) or just waiting enough (>15 min) while recording Ca^{2+} currents in whole-cell conditions in the absence of intracellular phosphorylating agents (Fedulova et al., 1985; Carbone & Lux, 1987b). HVA currents progressively disappeared, while T-type currents persisted in isolation. T-type currents could be nicely visualized by averaging five records at each potential and superimposing the averaged traces to appreciate the strict voltage dependence of T-type channel activation and inactivation (Carbone & Lux, 1987a) (Fig. 4b, right).

In addition, T-type channels are especially sensitive to block by Ni^{2+} ions while they are resistant to block by Cd^{2+} ions. HVA channels have opposing sensitivities (Fox et al., 1987; Narahashi et al., 1987). T-type channels are equally permeable to Ca^{2+}, Ba^{2+} and Sr^{2+}, while HVA channels are more permeable to Ba^{2+} and Sr^{2+} than Ca^{2+} ions (Bean, 1985; Fedulova et al., 1985). Interestingly, T-type channels carry also large Na^{+} currents in low $[Ca^{2+}]_o$ without losing its unique activation/inactivation characteristics (Fukushima & Hagiwara, 1985; Carbone & Lux, 1987a).

The Explosive Interest on T-Type Channels

The discovery of the T-type (LVA) channel at the soma of sensory neurons and cardiac myocytes of vertebrates, as well as the easiness to perform biophysical tests to isolate them from the rest of HVA channels, had great impact on the future studies of T-type Ca^{2+} channel function. It revealed a new component of the family of voltage-gated Ca^{2+} channels with a clearly different role on cell functioning with respect to HVA channels. It also stimulated the interest to uncover additional novel Ca^{2+} channel types in the non-somatic regions of neurons and in other excitable tissues. The consequence of this was an explosive rise of reports from 1985 on the existence of T-type channels either in isolation or together with L, N or other not yet identified HVA channels.

The existence of T-type channels was reported in nearly all excitable tissues: cardiac cells (Bean, 1985; Nilius et al., 1985; Mitra & Morad, 1986; Hagiwara et al., 1988), skeletal and smooth muscle (Bean et al., 1986; Loirand et al., 1986; Beam & Knudson, 1988), GH3 pituitary cell line (Armstrong & Matteson, 1985; Cohen & McCarthy, 1987), mouse neuroblastoma (Narahashi et al., 1987), various types of peripheral and central neurons (Bossu et al., 1985, 1989; Fedulova et al., 1985; Yaari et al., 1987; Coulter et al., 1989; Crunelli et al., 1989), pituitary cells (Cota, 1986; Marchetti et al., 1987), adrenal gland (Cohen et al., 1988; Bossu et al., 1991; Mlinar et al., 1993) and pancreatic β-cells (Ashcroft et al., 1990; Sala & Matteson, 1990). The existence of T-type channels was also soon extended to non-excitable cells, i.e. spermatogenic cells (Arnoult et al., 1998), cultured astrocytes (Barres et al., 1988) and oligodendrocytes (Blankenfeld Gv et al., 1992). For a more complete list of tissues expressing T-type channels, please see Perez-Reyes (2003).

It became also very clear that T-type channels could carry significant "window Ca^{2+} currents" near resting potential to regulate Ca^{2+} homeostasis near resting potential (Coulter et al., 1989; Williams et al., 1997b) and drive Ca^{2+}-dependent basal activities, such as cell cycle, proliferation, differentiation, transcription and apoptosis. It became soon evident that an increase of T-type channel expression was often associated with cancer development and/or progression (Panner & Wurster, 2006; Monteith et al., 2007) and reported in numerous types of tumour cells (see Dziegielewska et al., 2014; Zhang et al., 2014 and chapter "Voltage-Gated Calcium Channels as Key Regulators of Cancer Progression" by Brackenbury in this book). The high expression of LVA T-type channels in sensory neurons have also uncovered the key role of these channels in nociceptive signalling, specifically in the development and maintenance of neuropathic pains (Jagodic et al., 2007) (see also chapter "Voltage-Gated Calcium Channels in the Afferent Pain Pathway" by Gerald Zamponi in this book).

The modern view of the LVA T-type channel is that of a channel involved in many key physiological processes (see Nilius & Carbone, 2014 for a recent review). Obviously, the physiological roles of T-type channels are primary linked to their unique low-threshold activation, which produces Ca^{2+} influx in non-excitable cells at rest and triggers all-or-none APs by activating sodium and HVA calcium channels in excitable cells. These signals regulate key processes, like muscle contraction, AP conduction, epileptic discharges, neurotransmission, hormone secretion, gamete interaction, gene expression and associated pathologies (see the chapters "T-Type Calcium Channels in Epilepsy" by Terry Snutch and "Functional Role and Plasticity of Voltage Gated Calcium Channels in the Control of Heart Automaticity" by Matteo Mangoni in this book).

The Ca^{2+} Channel Family Growths

The N-Type Channel

The N-type channel, originally identified in sensory neurons (Nowycky et al., 1985), was not found in the heart (Nilius et al., 1985) and in skeletal muscles (Beam & Knudson, 1988). It was described as a rapidly inactivating and DHP resistant with a single-channel conductance intermediate between L- and T-type channels. Since neurotransmitter release was insensitive to DHPs but sensitive to the peptide toxin ω-conotoxin (ω-CTx) GVIA from *Conus geographus* (Olivera et al., 1991), N-type channels were soon proposed as neuron specific and specialized for the release of neurotransmitters (Perney et al., 1986; Hirning et al., 1988). In these experiments, however, it was unclear whether ω-CTx GVIA blocked only the N-type channel or both N- and L-type channels as suggested by (McCleskey et al., 1987). This uncertainty of the blocking selectivity of ω-CTx GVIA derived from the simplified definition of the N-type channel as a "rapidly inactivating" HVA channel (Fox et al., 1987).

As outlined above, many groups disagreed on whether N- and L-type channels could be uniquely separated from one another based on their inactivation kinetics. The N-type channel was postulated to be responsible for the "fast inactivating" HVA current, while the L-type channel was responsible for the "steady-state plateau" current. There was indeed clear evidence that this was not the case (Swandulla et al., 1991; Jones & Elmslie, 1992). In bullfrog sympathetic neurons, the N-type channel (90% N, 10% L) carries most of the Ca^{2+} current. However, these currents inactivate slowly and more than 50% of the current persists even after 1-s step depolarization (Jones & Marks, 1989). Obviously, these currents carry an inactivating and a non-inactivating component that derive almost exclusively from the N-type channel. The same was evident in PC12 cells (Plummer et al., 1989) and

sensory neurons (Aosaki & Kasai, 1989). Peter Hess and collaborators elegantly solved the issue by proposing that N-type channels were "slowly" (not "fast") inactivating channels and ω-CTx GVIA was the selective blocker of these channels (Plummer et al., 1989). After that, the general agreement was that N- and L-type currents were best identified using ω-CTx GVIA to block N-type channels and DHP antagonists or DHP agonists to either block or potentiate L-type channels. This new pharmacological approach allowed identifying the two HVA channels in combination or isolation in a variety of brain and peripheral neurons and other excitable cells (for a review see Catterall, 2011; Jurkovicova-Tarabova & Lacinova, 2019).

The P/Q-Type Channel

An additional HVA Ca^{2+} channel subtype with very slow inactivation and insensitive to both DHP and ω-CTx GVIA was soon identified in the Purkinje cells of cerebellum (Llinás et al., 1989). The new channel was named P-type (for Purkinje) and was found sensitive to the new peptide toxin of the venom from the American funnel web spider *Agelenopsis aperta*, ω-agatoxin (ω-Aga) IVA (Mintz et al., 1992). Another ω-Aga IVA-sensitive current component, which showed more rapid inactivation and had lower affinity for the toxin, was subsequently identified in cerebellar granule cells. The original proposal was that this new channel was a different HVA channel, named Q-type (Randall & Tsien, 1995). However, P- and Q-type pharmacological and biophysical properties were soon found to be recapitulated from different splicing of the same molecular entity (Bourinet et al., 1999). For simplicity, the channel was subsequently indicated as "P/Q-type" within the currently accepted terminology.

The R-Type Channel

After introduction of ω-Aga IVA as a P-type channel inhibitor, it was found that some neurons, including cortical, spinal cord and hippocampal CA1 neurons, had a "residual" Ca^{2+} current that was resistant to DHPs, ω-CTx GVIA and ω-Aga IVA (Mintz et al., 1992). This component of current could also be recognized using a new Conus peptide, ω-CTx MVIIC (Hillyard et al., 1992), which was found to block potently N- and P/Q-type channels (see McDonough et al., 1996) and was convenient to use in combination with the other Ca^{2+} channel blockers to uncover the "residual" component of HVA currents in brain neurons. The "residual" current persisting to all the available organic Ca^{2+} channel blockers was familiar to nearly all Ca^{2+} channelists at that time but it was Tsien's group (Zhang et al., 1993) that ultimately associated it with a newly-recognized cloned channel that received the name "R-type". This component of calcium current was soon separated into two R-channel subtypes (Forti et al., 1994). R-type channels were also found to be selectively blocked by the peptide SNX-482 derived from the tarantula *Hysterocrates gigas* (Newcomb et al., 1998). It became also clear that while L-type and T-type channels were expressed in a wide variety of cell, the N-, P-, Q- and R-type channels were most prominent in neurons (see Catterall, 2011).

From Ionic Currents to the Molecular Structure of Ca^{2+} Channels

In parallel with the biophysical identification of HVA channel types, several groups started purifying the Ca^{2+} channel components of skeletal muscle. Specifically, the DHP receptor (L-type) that is highly expressed in the T-tubules of skeletal muscles. The groups of Bill Catterall (USA), Franz Hofmann (Germany), Michel Lazdunski (France), Kevin Campbell (USA) and Hartmut Glossmann (Austria) were able to purify the DHP receptor complex that was composed by five protein subunits: α1, α2, β, δ and γ (Curtis & Catterall, 1984; Flockerzi et al., 1986; Hosey et al., 1987; Leung et al., 1987; Striessnig et al., 1987). The α1 subunit (190 kDa) contained the 1,4-DHP binding site and was identified as the pore-forming protein in association with a disulphide-linked α2δ dimer (170 kDa), an intra-

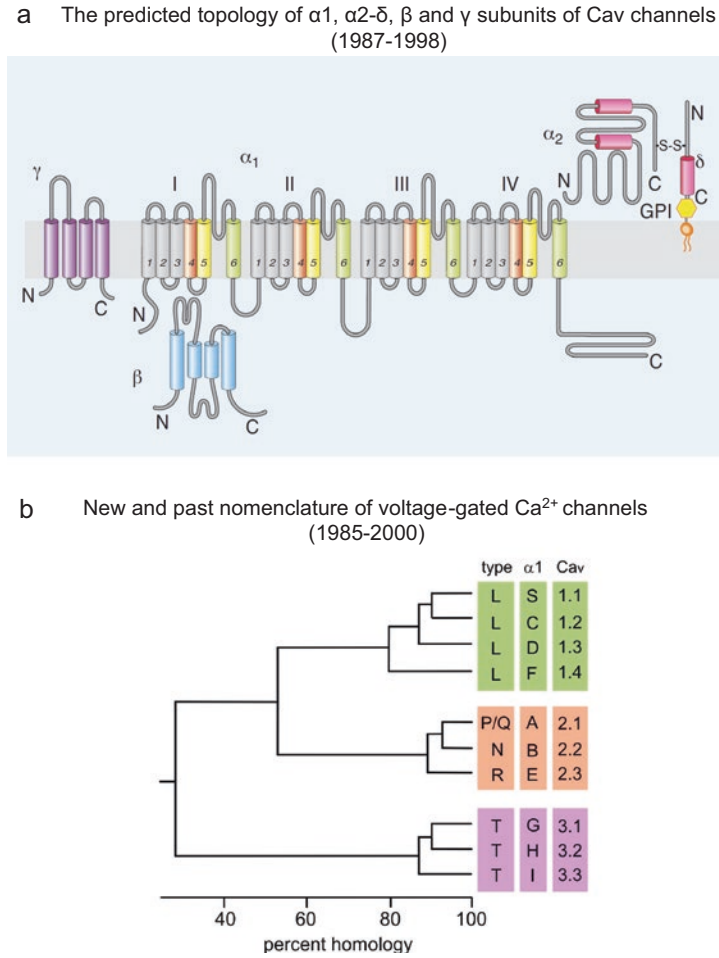

Fig. 5 Structure of Ca^{2+} channel subunits and classification of α1 subunits (1985–2000). (**a**) Subunit structure of Ca^{2+} channels. Predicted α helices are represented as cylinders. The lengths of lines correlate approximately to the lengths of the drawn polypeptide segments. The orange drawing on the δ subunit indicates the glycophosphatidylinositol (GPI) anchor to the membrane. (Redrawn after Catterall, 2011). (**b**) The three classifications used to indicate the ten isoforms of voltage-gated Ca^{2+} channels. The first (*type*) is based on biophysical properties and pharmacological sensitivity, while the last two (*α1* and *Cav*) are based on sequence homology. (Redrawn after Ertel et al., 2000)

cellular phosphorylated β subunit (55 kDa) and a transmembrane γ subunit (33 kDa) (Takahashi et al., 1987). Following this, Shosaku Numa's group in Japan was able to clone and sequence the cDNA of the skeletal muscle L-type channel α1 subunit (Tanabe et al., 1987). Through impressive team work, the group showed that by analogy with the previously cloned α1 subunit of the sodium channel (Noda et al., 1984), the amino acid sequence of the skeletal muscle α1 subunit (termed α1S) was organized into four homologous repeated domains (I–IV) with intracellular linkers and N- and C-terminals. Each domain contains six transmembrane segments (S1–S6) and a membrane-associated loop between transmembrane segments S5 and S6 (Fig. 5a).

This laid the foundation to clone and sequence other Ca^{2+} channel isoforms. The next was the cardiac L-type calcium channel, named α1C, that was cloned by homology with α1S (Mikami et al., 1989). Examining rat brain, Terry Snutch's group (Canada) next reported that multiple α1

subunits were expressed in the nervous system and encoded by distinct genes (Snutch et al., 1990). Designated α1A, α1B, α1C and α1D (the "Snutch" nomenclature – see Fig. 5b) were subsequently shown to encode P/Q-type (Mori et al., 1991; Starr et al., 1991; Bourinet et al., 1999), N-type (Dubel et al., 1992; Williams et al., 1992b) and two distinct L-type channels (Snutch et al., 1991; Williams et al., 1992a), respectively. The α1C subunit corresponding to the neuronal isoform of the previously cloned "cardiac" L-type. Curiously, the neuronal α1D isoform (Williams et al., 1992a) activated at more negative potentials and inactivated more slowly than the α1C subunit (Koschak et al., 2001; Xu & Lipscombe, 2001). A third neuronal L-type channel was later identified in the retina and named α1F (Strom et al., 1998). The channel had properties that were quite distinct from the α1D and α1C L-type isoforms (Koschak et al., 2003).

Snutch's group cloned also the α1E channel (Soong et al., 1993), which was classified as a LVA channel type, but soon became clear that it did not possess all the expected biophysical properties but rather those of the distinct R-type channel (Zhang et al., 1993). The last Ca^{2+} channels to be cloned were the T-type channels. Following an in silico approach Ed Perez-Reyes's group was able to clone the α1G, α1H and α1I subunits of rat brain (Perez-Reyes et al., 1998; Lee et al., 1999) and human heart (Cribbs et al., 1998). The cloning of T-type channels occurred after more or less 14 years from the 1984 functional discovery of the LVA T-type channel. It also ended the misconception of some colleagues that the T-type Ca^{2+} channel was a possible artefact of some sort. Following the cloning and initial study of all the calcium channel α1 subunits identified in the mammalian genome, a rationalized nomenclature was adopted in 2000, grouping the α1 subunits into Cav1 (L-type), Cav2 (N, P/Q, R-type) and Cav3 (T-type) (Ertel et al., 2000) (Fig. 5b). Since then, the distinctive pharmacological, biophysical and modulatory properties of alternative splicing of the α1 subunits have also been recognized.

Recent advances in the structural studies of calcium channels using X-ray crystallography and cryo-electron microscopy (cryo-EM) have brought new unprecedented insights into the molecular basis of their function and pharmacology. The first crystal structure for a Ca^{2+}-selective voltage-gated channel was obtained using the homotetrameric bacterial Nav channel (NavAb), which was mutated at the selectivity filter to form a Ca^{2+}-selective pore (CavAb) (Payandeh et al., 2011). The bacterial CavAb channel provided new insights into the mechanism of Ca^{2+} permeation (Tang et al., 2014) and binding locations of different calcium channel antagonists (Tang et al., 2016). Thanks to major progresses in cryo-EM technology, it is now available also a high-resolution 3D structure (3.6 Å) of the pore and the subunit arrangement of the rabbit skeletal muscle Cav1.1 channel (Wu et al., 2016). Following the same approach, the human Cav3.1 (T-type) and Cav2.2 (N-type) channel structures are also accessible for function and pharmacological studies (Zhao et al., 2019; Gao et al., 2021). This opens unexpected avenues on the function of Cav channel subunits. For more details on Cav channels structure, please refer to chapter "Pharmacology of Voltage-Gated Calcium Channels at Atomic Resolution" by Bill Catterall in this book.

The cAMP-Mediated Enhancement of Cardiac L-Type Channels as First Example of Ca^{2+} Channel Modulation

As for other voltage-gated ion channels, HVA Ca^{2+} channels are effectively up- or down-regulated by a variety of neurotransmitter-mediated second messenger pathways. Of the many modulatory pathways acting on Ca^{2+} channels, the first one discovered was the cAMP-dependent protein phosphorylation pathway that mediates the up-regulatory action of adrenaline (A) and noradrenaline (NA) on cardiac L-type channels. Both catecholamines were shown to cause an elevation and increased duration of the cardiac AP as a consequence of a marked potentiation of the Ca^{2+} current amplitudes (Reuter, 1967; Vassort et al., 1969). The action was mimicked either by injecting cAMP into myocardial fibres (Tsien et al., 1972), applying mono- or dibutyryl-cAMP analogues, phosphodiesterase

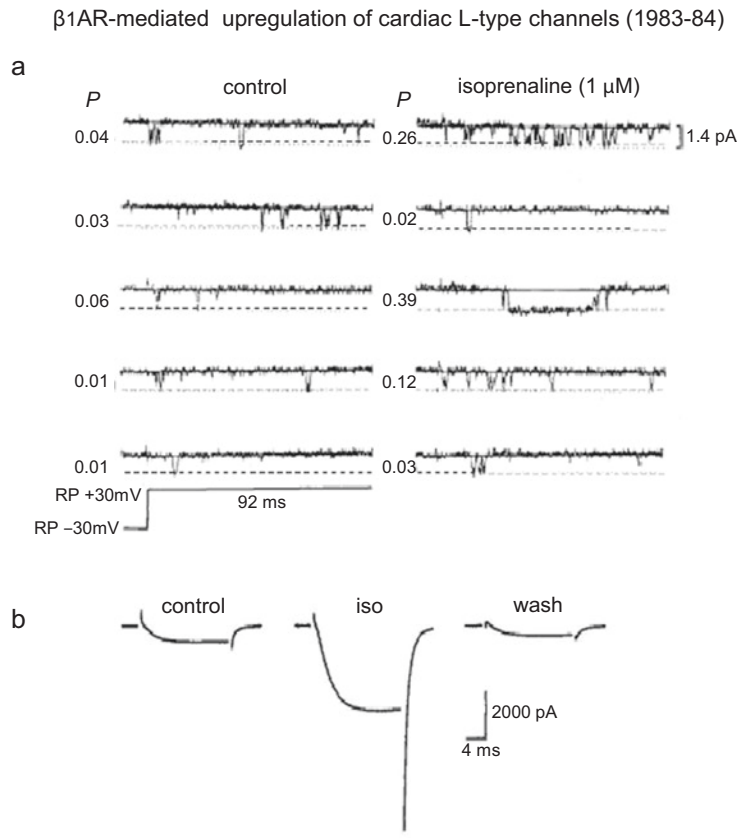

Fig. 6 The β_1-AR-agonist isoprenaline increases the size of cardiac L-type currents by increasing the channel open probability (1983–1984). (**a**) Isoprenaline (1 μM) increases the open probability (*P*) of single L-type channels of cultured rat heart cells, while the current amplitude of the single event remained the same (Ba^{2+} as charge carrier). (Redrawn with permission from Reuter, 1983). (**b**) Isoprenaline (0.5 μM) increases about fivefold the size of L-type currents recorded at +10 mV from frog ventricular heart cells (Vh −60 mV). (Redrawn with permission from Bean et al., 1984)

inhibitors, or by injecting the catalytic subunit of cAMP-dependent protein kinase (PKA) (see Catterall, 2000 for a review).

Harald Reuter was the first to demonstrate that single-cardiac L-type channel activity is potentiated by cAMP analogues and β1-adrenoreceptor (β1-AR) agonists (Cachelin et al., 1983) (Fig. 6a). Addition of isoprenaline or 8-bromo-cAMP to the bath up-regulated the cardiac L-type channel activity by increasing the open channel probability (*P*) while preserving the size of single-channel conductance (Reuter, 1983). The action included also a three-fold increase in the number of active L-type channels, contributing to the impressive increase of the control current at +10 mV induced by isoprenaline (Fig. 6b) (Bean et al., 1984). These early findings identified the main role that the β1-AR-mediated up-regulation of cardiac L-type channels plays in the control of the "fight-or-flight" response of body function.

The experiments on cAMP-mediated enhancement of cardiac DHP-sensitive channels were soon extended to skeletal and smooth muscles, endocrine cells and neurons, where the distinctive up-regulatory effects of the cAMP/PKA phosphorylation pathway on L-type channels were observed at different degrees (see Armstrong et al., 1991 for a review). In the following years a multitude of second messenger pathways were discovered with a more or less directed up- or down-regulatory action on L-type, as well as on N-, P/Q- and R-type channels in excitable tissues. This made more complex and intriguing to understand how extracellular or intracellular second messengers modulate Ca^{2+} channels. Remarkably, recent work has shown that the cAMP-mediated enhancement of the cardiac calcium channel does not involve phosphorylation of the channel itself, as was long assumed, but rather a small interacting Gem ATPase that constitutively inhibits channel activation (Liu et al., 2020). Two chapters in

this book cover the recent achievements on transmitter modulation of calcium channels (see chapters "Modulation of VGCCs by G-protein Coupled Receptors and Their Second Messengers" by Herlitze et al. and "Calmodulin Regulation of Voltage-Gated Calcium Channels" by Dick and Ben Johny).

Early Observations on the GPCR-Mediated Inhibition of Neuronal Ca2+ Channels

Nearly in parallel with the discovery of the β1-AR-mediated potentiation of cardiac L-type channels, Kathleen Dunlap and Gerald Fischbach (USA) reported a new completely different modulatory action of NA on neuronal HVA channels. They showed that NA reduces the duration of APs in chick dorsal root ganglion cells (Dunlap & Fischbach, 1978) as a consequence of a reduction of HVA Ca^{2+} currents (Dunlap & Fischbach, 1981) (Fig. 7a, left). The same occurred with serotonin and GABA. The inhibitory action of these neurotransmitters was proposed as a possible mechanism for presynaptic inhibition and attracted immediately the attention of many researchers interested on the function of presynaptic Ca^{2+} channels. Subsequent findings confirmed and extended these observations to rat sympathetic neurons (Galvan & Adams, 1982), suggesting a marked selectivity of the NA-mediated action for neuronal HVA channels but no requirements of cAMP, cGMP or Ca^{2+} as second messengers (Forscher et al., 1986). In the following years (1986–1987) several groups were able to show that NA and other neurotransmitters inhibited the HVA Ca^{2+} currents, but it was not clear whether the action was on N- and L-type channels as the currents of the two channels were not yet clearly biophysically separated (see above).

Carla Marchetti, Dieter Lux and I were first to observe that dopamine (DA) and NA inhibited the HVA channels of sensory and sympathetic avian neurons in a "voltage-dependent" manner (Marchetti et al., 1986). DA and NA were found to produce a dramatic slowdown of HVA channel activation at low membrane potentials that was accelerated and partly relieved at higher membrane depolarizations (Fig. 7a, middle). Similar findings were reported by other groups using leu-enkephalin and somatostatin (Luini et al., 1986; Tsunoo et al., 1986; Ikeda et al., 1987).

We suggested that the slowdown of HVA channel activation was a consequence of the slow opening of HVA channels that were inhibited at rest and slowly unblocked while opening during mild depolarizations. Increasing depolarization accelerated the unblock rate, so that quickly repeated depolarizations were predicted to remove the block and recover the fast time course of HVA channel activation. This interpretation was in contrast with the more popular interpretation (at that time) that the inhibitory action of neurotransmitters was due to a "block" of a fast inactivating N-type current while preserving a

Fig. 7 (continued) (Redrawn with permission from Dunlap & Fischbach, 1978, 1981). Center, dopamine (DA, 10 μM) slows down the activation of HVA Ca^{2+} currents in 5 mM Ca^{2+}in chick DRG neurons. Activation of HVA currents accelerates with DA by increasing the step depolarization from 0 to +10 mV (Vh −70 mV). Calibration: 0.7 nA, 10 ms. (Redrawn with permission from Marchetti et al., 1986). Right, slow activation of HVA Ca^{2+} currents induced by GTP-γ-S (500 μM) at +10 mV (Vh −80 mV) in rat sensory neurons. Calibrations: top (50 pA, 10 ms), bottom (0.5 nA, 25 ms). (Redrawn with permission from Dolphin & Scott, 1987). (**b**) Left, the tails of Ca^{2+} currents at −50 mV after step depolarization to +10 mV are strongly reduced by NA (30 μM), while are fully preserved after step depolarization to +110 mV in bullfrog DRG neurons. *Inset:* the "willing-reluctant" model proposed by Bean (1989) with inclusion of the voltage-independent RO↔O transition introduced by Elmslie et al. (1990) to account for the pre-pulse induced "facilitation". (Redrawn with permission from Bean, 1989). Right, the "pre-pulse protocol" used to prove that reluctant N-type channels bound to G protein subunits recover their fast and complete activation by anticipating the test depolarization to 0 mV with a strong pre-pulse to +70 mV in bullfrog sympathetic neurons. (Redrawn with permission from Elmslie et al., 1990). (**c**) Left, prolonged delay of first openings during sequential recordings of single-N-type channel traces at +20 mV in control (left) and in the presence of NA (20 μM) + DPDPE (1 μM) (right) (Vh = −90 mV). Right, facilitation of single-N-type channel activity by a strong pre-pulse in the presence of NA + DPDPE. Same conditions as in the left panel. In most traces the delayed openings induced by the two agonists (left) were accelerated by the pre-pulse to +120 mV (right). (Redrawn with permission from Carabelli et al., 1996)

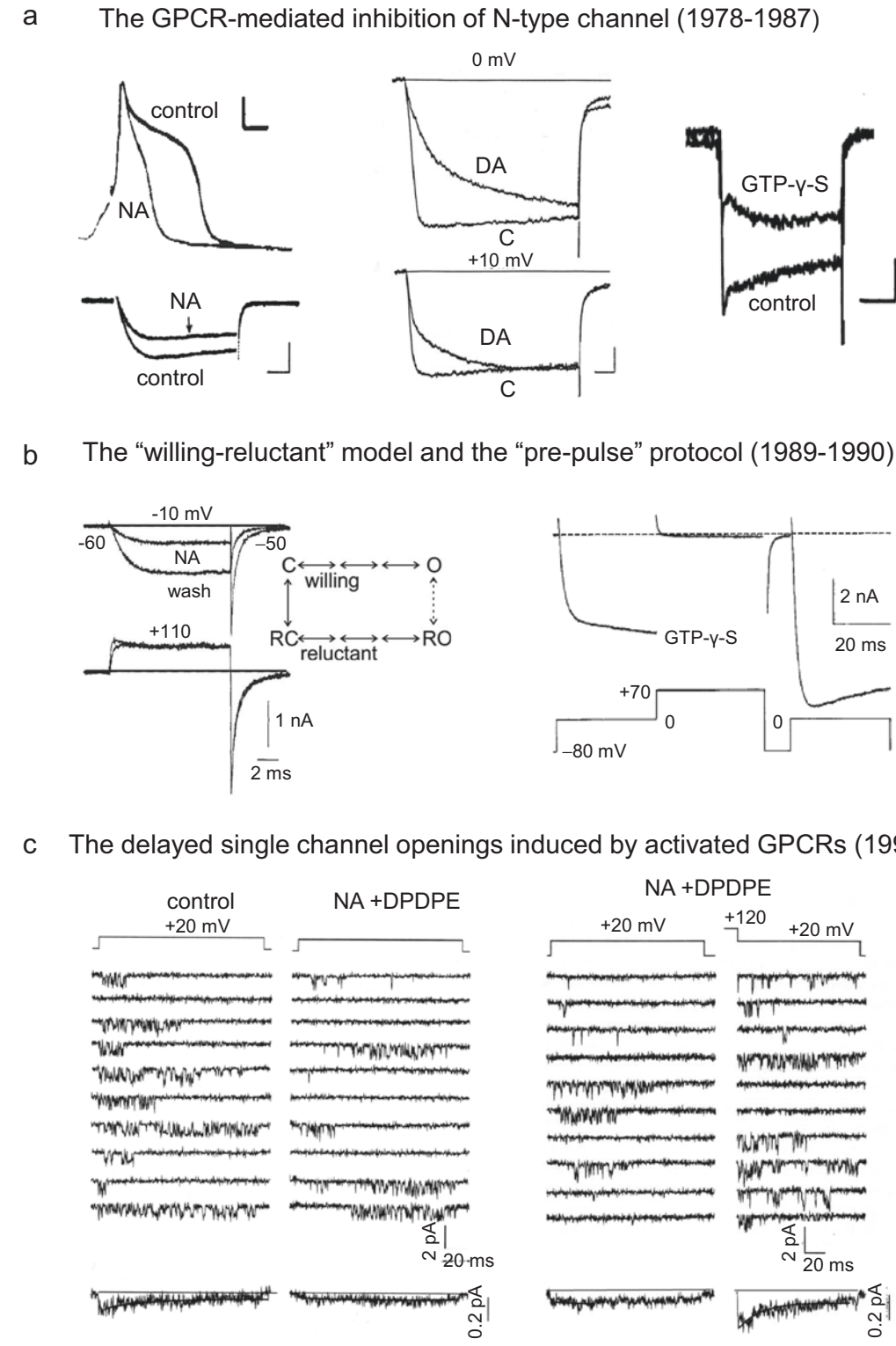

Fig. 7 The GPCR-mediated inhibition of N-type channels (1978–1996). (**a**) Left, NA (100 μM) shortens the duration of APs and reduces the Ca^{2+} currents in chick DRG neurons (10 mM Ca^{2+} and TTX in the bath, TEA in the pipette). Step depolarization to +60 mV from Vh −50 mV. Calibrations: top (20 mV, 2 ms), bottom (10 nA, 5 ms).

slowly activating L-type component (Dolphin & Scott, 1987; Gross & Macdonald, 1987; Wanke et al., 1987; Lipscombe et al., 1989).

While this controversy continued, evidence increased rapidly concerning the involvement of intracellular GTP-binding proteins through the activation of G-protein-coupled receptors (GPCRs) by neurotransmitters. Annette Dolphin's (UK) and Kathleen Dunlap's (USA) groups were among the first to prove that the non-hydrolysable GTP analogue, GTP-γ-S, mimics the inhibitory action of neurotransmitters (Dolphin & Scott, 1987) (Fig. 7a, right), while the non-hydrolysable GDP analogue, GDP-β-S, prevents the inhibition (Holz et al., 1986; Wanke et al., 1987). Ca^{2+} channel modulation by neurotransmitter and GTP-γ-S was prevented by cell incubation with pertussis toxin (PTX) (Holz et al., 1986; Lewis et al., 1986), while intracellular application of purified G protein subunits restored the GPCR-mediated response to PTX-treated cells (Toselli et al., 1989).

Towards a Full Understanding of the GPCR-Induced Delayed Activation of HVA Channels

In the years 1986–1989, many groups worked on the inhibitory action of G proteins on HVA channels and confirmed that the mechanism mediated by PTX-sensitive G proteins was unique in regulating the inhibitory action of most neurotransmitters in nearly all neurons tested (see Hille, 1994; Dolphin, 2003 for reviews). There was, however, no clear explanation of the molecular mechanism by which G proteins altered the kinetics and voltage dependence of HVA channel activation. Bruce Bean (USA) built on the previously observed "voltage-dependent" change in kinetics to propose an intuitive model of "reluctant" versus "willing" modes of channel activation based on a "positive shift" of the voltage dependence of tail currents in the presence of neurotransmitters (inset in Fig. 7b, left) (Bean, 1989). In the normal "willing" gating mode (C ↔ O) channels can be readily opened by small or moderate depolarizations while in the "reluctant" gating mode induced or stabilized by transmitters (RC ↔ RO), channels were postulated to require much larger depolarizations to activate. Using NA and bullfrog DRG neurons, Bean showed that the tail currents of N-type currents at −50 mV were strongly reduced following mild depolarizations (−10 mV) but fully recovered after strong depolarization to +110 mV (Fig. 7b, left). Assuming a voltage-independent equilibration between the two modes (RC ↔ C), the model could mimic the slow activation of HVA channels and supported the idea that neurotransmitters produce a "resting inhibition" that can be "slowly reversed" during depolarization rather than a "permanent block" of transiently inactivating channels.

However, the fully convincing evidence that the "delayed activation" of HVA channels derives from an "unblock" of G protein-inhibited channels at rest came from the work of Keith Elmslie and Steve Jones (USA). Using bullfrog sympathetic neurons, Keith and Steve showed that if moderate depolarizations to 0 mV were preceded by a strong pre-pulse depolarization to +70 mV, the HVA current inhibited by the luteinizing hormone-releasing hormone (LHRH) could fully recover its fast activation (Fig. 7b, right) (Elmslie et al., 1990). To account for this, it was necessary to include the voltage-independent transition RO ↔ O in the "willing-reluctant" model (dashed arrows in the inset of Fig. 7b, left).

As mentioned in the introduction of their Neuron paper, Keith and Steve nicely commented on the idea Hans Dieter Lux, Carla Marchetti and I had in our 1986 paper. They wrote, "Marchetti et al. (1986) speculated that the slow activation results from voltage-dependent removal of transmitter block. If this were the case, a strong depolarizing pulse should remove inhibition, returning the current to normal. We find that this does occur ….". Two issues became evident from the new data of Elmslie et al. (1990). First, as sympathetic neurons express primarily N-type channels (95% of the total), it was unequivocal that G protein subunits acted effectively on N-type channels. Second, the "pre-pulse protocol" used to test the existence of the voltage-dependent effects of neurotransmitter-induced inhibition was so ingenious and easy to apply that became immediately the routine test used by all groups to distinguish

"voltage-dependent" from "voltage-independent" GPCR-mediated inhibitions. Indeed, a voltage-independent mechanism was shown to coexist in sympathetic (Bernheim et al., 1991) and sensory neurons (Luebke & Dunlap, 1994) but, at variance with the voltage-dependent one, it was mediated by soluble second messenger pathways (see Tedford & Zamponi, 2006 for review).

A voltage-dependent inhibition by neurotransmitters was soon reported also for the P-type channels of Purkinje neurons (Mintz & Bean, 1993), suggesting that the voltage-dependent relief of the inhibition (also termed "facilitation") is physiologically relevant at presynaptic terminals where N- and P/Q-type channels are highly expressed (see for review Tedford & Zamponi, 2006). Indeed, presynaptic facilitation was shown to occur at various degrees when delivering high-frequency trains of AP-like waveforms, giving a functional role to the G protein-mediated inhibition of presynaptic Ca^{2+} channels (Womack & McCleskey, 1995; Brody et al., 1997; Williams et al., 1997a), which likely underlies the widespread phenomenon of presynaptic inhibition of synaptic transmission by G-protein-coupled transmitter receptors.

Looking Deeper to the Structure and Function of Cav2 Channels Modulation by G proteins

Having clarified how G proteins inhibit N- and P/Q-type channels, the next issue was to identify which G protein subunit (α or βγ) was more effective on binding to the α1 subunit of Cav2 channels. Steve Ikeda (USA) and Bill Catterall with Bertil Hille (USA) were able to demonstrate independently that only the Gβγ dimer was involved in this modulation (Ikeda, 1996) and that the Gβγ subunit was effective on both N- and P/Q-type channels (Herlitze et al., 1996). Following this, the subsequent issue was to identify the α1 subunit regions to which the Gβγ dimer binds during the closed state of the channel. Terry Snutch's group showed both that a single Gβγ dimer interacts with the N-type channel to affect modulation (Zamponi & Snutch, 1998) and that Gβγ physically binds to the α1 subunit I-II linker (Zamponi et al., 1997). Annette Dolphin's (UK) and Lutz Birnbaumer (USA) groups further postulated that the N-terminal (Page et al., 1998) and the C-terminal (Qin et al., 1997) also represent potential intracellular regions of the α1 subunit of the channel where the Gβγ dimer could bind. Curiously enough, the intracellular I-II linker contains the highly conserved motif AID (α-interaction domain), to which the β subunit isoforms bind to regulate the α1 subunit (Pragnell et al., 1994). Thus, it was soon evident that the presence or absence of β subunits was an important determinant to up- or down-regulate the Gβγ-mediated inhibition of Cav2 channels (N, P/Q, R) (Bourinet et al., 1996; see Tedford & Zamponi, 2006; Zamponi & Currie, 2013; Dolphin, 2018 for reviews).

The modern view of G proteins inhibition is now enriched by further structural details. They include a better identification of the regions of the AID motif interacting with the β and Gβγ subunits, and the crosstalk with other cell signalling pathways, synaptic proteins and γ subunits. See chapter "Modulation of VGCCs by G-protein Coupled Receptors and Their Second Messengers" by S. Herlitze et al. in this book for a review on these issues.

In parallel to this, several groups were trying to identify the origin of the slowdown of Cav2 channel activation at the single-channel level. The prediction was that after GPCR activation, the inhibited N-type channels would have delayed their first opening (first latency) by a certain amount and strong depolarizing pre-pulses would have prevented the delay, recruiting the fast channel opening of control. Valentina Carabelli and I in Torino (Italy) were the first to show that slowdown of N-type channel activation by NA, and the δ-opioid agonist DPDPE in IMR32 cells was primarily due to a marked delay of the first single-channel opening with a consequent increase of the first latency of openings at moderate depolarization (Carabelli et al., 1996) (Fig. 7c, left). The delay had no effect on single-channel conductance, had minor effects on the mean open time and was effectively removed (facilitated) by large depolarizations (Fig. 7c, right). Several months

later, similar findings were reported also by David Yue's group (USA) using HEK 293 cells transfected with recombinant M2 muscarinic receptors and N-type channels, confirming that an augmented latency of first opening was the cause of the delayed first openings of reconstituted N-type channel (Patil et al., 1996). Yue's and Elmslie's groups went further and showed that the N-type channel of sympathetic neurons (Lee & Elmslie, 2000) or transfected HEK 293 cells (Colecraft et al., 2001) exhibit "reluctant openings" that appeared as rare single brief openings during the low open probability interval that preceded the "willing openings". These later appeared in the form of repeated wide openings with increased open probability. Reluctant openings, however, were absent in reconstituted P/Q--type channels (Colecraft et al., 2001). Hence, after about 15 years, these studies settled ultimately the controversy concerning the molecular mechanisms by which the Gβγ dimer delays the activation of Cav2 channels by directly binding to the α1 subunit. GPCRs can also recruit several other distinct mechanisms, including phosphorylation, lipid signalling pathways and channel trafficking, that result in voltage-independent inhibition (for a review see Zamponi & Currie, 2013, chapters "Modulation of VGCCs by G-protein Coupled Receptors and Their Second Messengers" by Herlitze et al. and "Trafficking of Neuronal Calcium Channels" by Norbert Weiss in this book).

Take-Home Message

The first consideration, after having reviewed only a small part of the immense literature on voltage-gated Ca^{2+} channels (>50,000 papers; PubMed), is on the impressive work done by many groups over the past 50 years to take us to the present knowledge of the structure and function of Ca^{2+} channels. Thanks to the outstanding achievements of hundreds of scientists working in many countries, learning from and inspiring each other by papers and conference presentations, Cav channels are now widely recognized as key regulatory molecules of many vital functions of the brain, sensory and motor neurons, heart, skeletal and smooth muscles and neuroendocrine and glial cells, as well as many vital functions of non-excitable cells (see Pitt et al., 2021). Ca^{2+} channels are now recognized as the targets of an increasing number of human pathologies, which derive either directly from genetic mutations of the channel subunits (α1, α2δ, β) (Ca^{2+} channelopathies) or indirectly from the altered second messenger pathways that up- or down-regulate Ca^{2+} channel function. All these new studies are in progress and described in details in this book.

A second consideration is on how the present status of Ca^{2+} channels has been reached thanks to the availability of new technical advances that occurred periodically and to the outstanding work of the many groups who exploited them to achieve milestone discoveries. As in any field of science, new advances created disputes and controversies that required time to be settled. Regardless of being right or wrong, it was crucial to participate to the debates that systematically led to new advances.

A third consideration is more personal and concerns how important it was for me to be at the right time and in the right place. Joining Lux's group at the MPI of Neurophysiology in Munich offered me the unique opportunity to deal with key unsolved issues on Ca^{2+} channel function that were completely open at that time and to discuss them with the leading personalities in the field at that moment. It was a great pleasure to have met and discussed with most of them, including those who are no longer with us: Roger Eckert, Susumu Hagiwara, Hans Dieter Lux, Platon Kostyuk, Wolfang Trautwein and David Yue.

Finally, I hope that this brief history towards the understanding of Ca^{2+} channel function will help young and new investigators realize and appreciate the pioneering work done by the many colleagues who worked with strength and passion on Ca^{2+} channel structure and function.

Acknowledgements I wish to thank Bruce Bean, Terry Snutch, Keith Elmslie and David Vandael for reading, correcting and commenting enthusiastically on the manuscript. I wish also to thank Valentina Carabelli, Andrea

Marcantoni and Giulia Tomagra for continuous support and discussions, and the editors Gerald Zamponi and Norbert Weiss for suggestions and encouragements.

References

Aosaki, T., & Kasai, H. (1989). Characterization of two kinds of high-voltage-activated Ca-channel currents in chick sensory neurons. Differential sensitivity to dihydropyridines and omega-conotoxin GVIA. *Pflügers Archiv/European Journal of Physiology, 414*, 150–156.

Armstrong, C. M. (1966). Time course of TEA(+)-induced anomalous rectification in squid giant axons. *The Journal of General Physiology, 50*, 491–503.

Armstrong, C. M. (1969). Inactivation of the potassium conductance and related phenomena caused by quaternary ammonium ion injection in squid axons. *The Journal of General Physiology, 54*, 553–575.

Armstrong, C. M., & Matteson, D. R. (1985). Two distinct populations of calcium channels in a clonal line of pituitary cells. *Science (New York, N.Y.), 227*, 65–67.

Armstrong, D. L., Rossier, M. F., Shcherbatko, A. D., & White, R. E. (1991). Enzymatic gating of voltage-activated calcium channels. *Annals of the New York Academy of Sciences, 635*, 26–34.

Arnoult, C., Villaz, M., & Florman, H. M. (1998). Pharmacological properties of the T-type Ca2+ current of mouse spermatogenic cells. *Molecular Pharmacology, 53*, 1104–1111.

Ashcroft, F. M., Kelly, R. P., & Smith, P. A. (1990). Two types of Ca channel in rat pancreatic beta-cells. *Pflügers Archiv/European Journal of Physiology, 415*, 504–506.

Baker, P. F., Hodgkin, A. L., & Ridgway, E. B. (1971). Depolarization and calcium entry in squid giant axons. *The Journal of Physiology, 218*, 709–755.

Baker, P. F., Meves, H., & Ridgway, E. B. (1973). Calcium entry in response to maintained depolarization of squid axons. *The Journal of Physiology, 231*, 527–548.

Barres, B. A., Chun, L. L., & Corey, D. P. (1988). Ion channel expression by white matter glia: I. Type 2 astrocytes and oligodendrocytes. *Glia, 1*, 10–30.

Beam, K. G., & Knudson, C. M. (1988). Calcium currents in embryonic and neonatal mammalian skeletal muscle. *The Journal of General Physiology, 91*, 781–798.

Bean, B. P. (1985). Two kinds of calcium channels in canine atrial cells. Differences in kinetics, selectivity, and pharmacology. *The Journal of General Physiology, 86*, 1–30.

Bean, B. P. (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature, 340*, 153–156.

Bean, B. P., Nowycky, M. C., & Tsien, R. W. (1984). Beta-adrenergic modulation of calcium channels in frog ventricular heart cells. *Nature, 307*, 371–375.

Bean, B. P., Sturek, M., Puga, A., & Hermsmeyer, K. (1986). Calcium channels in muscle cells isolated from rat mesenteric arteries: Modulation by dihydropyridine drugs. *Circulation Research, 59*, 229–235.

Bernheim, L., Beech, D. J., & Hille, B. (1991). A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. *Neuron, 6*, 859–867.

Berridge, M. J., Bootman, M. D., & Roderick, H. L. (2003). Calcium signalling: Dynamics, homeostasis and remodelling. *Nature Reviews. Molecular Cell Biology, 4*, 517–529.

Blankenfeld Gv, G. V., Verkhratsky, A. N., & Kettenmann, H. (1992). Ca2+ channel expression in the oligodendrocyte lineage. *The European Journal of Neuroscience, 4*, 1035–1048.

Bossu, J. L., Feltz, A., & Thomann, J. M. (1985). Depolarization elicits two distinct calcium currents in vertebrate sensory neurones. *Pflügers Archiv/European Journal of Physiology, 403*, 360–368.

Bossu, J. L., Dupont, J. L., & Feltz, A. (1989). Calcium currents in rat cerebellar Purkinje cells maintained in culture. *Neuroscience, 30*, 605–617.

Bossu, J. L., De Waard, M., & Feltz, A. (1991). Two types of calcium channels are expressed in adult bovine chromaffin cells. *The Journal of Physiology, 437*, 621–634.

Bourinet, E., Soong, T. W., Stea, A., & Snutch, T. P. (1996). Determinants of the G protein-dependent opioid modulation of neuronal calcium channels. *Proceedings of the National Academy of Sciences of the United States of America, 93*, 1486–1491.

Bourinet, E., Soong, T. W., Sutton, K., Slaymaker, S., Mathews, E., Monteil, A., Zamponi, G. W., Nargeot, J., & Snutch, T. P. (1999). Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. *Nature Neuroscience, 2*, 407–415.

Brehm, P., & Eckert, R. (1978). Calcium entry leads to inactivation of calcium channel in Paramecium. *Science (New York, N.Y.), 202*, 1203–1206.

Brody, D. L., Patil, P. G., Mulle, J. G., Snutch, T. P., & Yue, D. T. (1997). Bursts of action potential waveforms relieve G-protein inhibition of recombinant P/Q-type Ca2+ channels in HEK 293 cells. *The Journal of Physiology, 499*(Pt 3), 637–644.

Brown, A. M., Camerer, H., Kunze, D. L., & Lux, H. D. (1982). Similarity of unitary Ca2+ currents in three different species. *Nature, 299*, 156–158.

Cachelin, A. B., de Peyer, J. E., Kokubun, S., & Reuter, H. (1983). Ca2+ channel modulation by 8-bromocyclic AMP in cultured heart cells. *Nature, 304*, 462–464.

Carabelli, V., Lovallo, M., Magnelli, V., Zucker, H., & Carbone, E. (1996). Voltage-dependent modulation of single N-type Ca2+ channel kinetics by receptor agonists in IMR32 cells. *Biophysical Journal, 70*, 2144–2154.

Carafoli, E., Santella, L., Branca, D., & Brini, M. (2001). Generation, control, and processing of cellular cal-