Functional and molecular characterization of a novel afterdepolarizing current in hippocampal pyramidal neurons

Thesis submitted at UCL

by

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for the degree of

Doctor of Philosophy, PhD
Declaration

I, Marisol Sampedro Castañeda confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

_________________________
Abstract

In the hippocampus, pyramidal neuron excitability can be regulated in a fast and dynamic manner during the acquisition and integration of information. In particular, firing patterns are shaped by the combination of coincident inputs and intrinsic electrical properties of the cell. Afterpotentials represent one such intrinsic mechanism whereby the simultaneous activation of Ca$^{2+}$- and voltage-gated conductances following firing activity determines the membrane potential of the neuron for up to several seconds and influences further spike output. In the present study, the use of whole-cell patch clamp electrophysiology and pharmacology reveals the occurrence of a novel afterdepolarizing (ADP) mixed-cationic current in cultured hippocampal neurons and slices. This conductance, named $I_{ADP}$, has a time course in the order of hundreds of milliseconds and can be visualized upon pharmacological suppression of the afterhyperpolarizing (AHP) currents mediated by SK and KCNQ channels and underlying the medium afterhyperpolarization (mAHP) in these neurons. $I_{ADP}$ is activated by intracellular Ca$^{2+}$ rises as demonstrated using BAPTA in the pipette solution, is carried mainly by Na$^+$ ions and is positively modulated by temperatures in the physiological range. The pharmacological profiling of $I_{ADP}$ indicates that a transient receptor potential channel (TRP) mediates a component of this current. In particular, the actions of the specific TRPM2 channel agonist adenosine diphosphate ribose (ADPR), in both cultured neurons and acute slices, point at the involvement of this cationic channel in the generation of a post-spike depolarization in these neurons. This result was corroborated by evidence from a heterologous expression system and confirmed in the culture preparation by genetic manipulation of the functional expression of TRPM2 channels using a dominant negative mutant subunit. Finally, experiments in
hippocampal slices using the specific agonist ADPR demonstrate that, under physiological conditions, $I_{ADP}$ indeed coexists and interacts with currents underlying the mAHP and that they act in unison to regulate early spike frequency adaptation in CA1 pyramidal cells. Thus, TRPM2 channels mediate a Ca$^{2+}$-activated cationic current in hippocampal pyramidal cells, which contributes, at least in part, to a medium duration ADP with important implications for the regulation of neuronal firing activity.
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Chapter One

Introduction
Over the years, the hippocampus has been a focus of intense biomedical research owing to its propensity to suffer ischemic damage and be the centre of the generation of various types of epilepsy (Lipton, 1999; McCormick and Contreras, 2001). This philogenetically old cortical structure is now recognized to play an important role in the acquisition and consolidation of memory and the control of attention. In particular, the hippocampal circuitry is crucially involved in the development of space awareness and orientation (Anderson et al., 2007). Its exquisitely defined cytoarchitectural organization (Fig. 1A) allows relative ease of access to individual components of the network with electrodes, which has greatly facilitated studies in brain sections and in intact animals.

Within the hippocampus, pyramidal and granule cells constitute the great majority of the neuronal types, with inhibitory interneurons making up the remaining cell population. Histologically, pyramidal and granule cell bodies are disposed in tight arrays forming the so-called cornu Ammonis and dentate gyrus, respectively (Fig. 1A, CA and DG). The former region is further subdivided into three subfields according to particular patterns of density and innervations of the pyramidal cells (CA1-3). CA1 neurons receive excitatory inputs from CA3 cells, the enthorinal cortex and the thalamus, and are also laterally connected. Inhibitory interneurons form synapses onto their triangular cell body, specific dendritic domains and the axon, which stems from the base of the soma (Amaral and Lavenex, 2007). These innervations, together with the intrinsic electrical properties of these complex cells, give rise to distinctive firing modes within the population of CA1 pyramidal cells.

The vast majority of pyramidal neurons in the hippocampus exhibit a regular firing pattern, responding with a single spike to brief supra-threshold stimulation
Figure 1. Hippocampal pyramidal neurons and intrinsic excitability. A) Schematic representation of a transverse section of the rat hippocampus illustrating the main histological subdivisions of the hippocampus proper (CA: cornu Ammonis, cell bodies of pyramidal neurons; DG: dentate gyrus, granule cell bodies) and simplified circuitry. pp: perforant path, axonal projections from entorhinal cortex; fi: fimbria, main output from the hippocampus (from Anderson et al., 2007). B) Typical firing pattern of a hippocampal pyramidal neuron from the CA1 region; trace illustrates progressive reduction in number of spikes fired (SFA) in response to sustained somatic current injection. Two distinct phases of SFA can be distinguished: early (~ first 300 ms) and late (>300 ms). C) Afterhyperpolarizations following action potential repolarization and spike accommodation (from Sah, 1996). D) Fast (tens of milliseconds; left) and slow (seconds; right) after-depolarizing potentials in these same neurons in the absence (fADP) and presence (sADP; Fraser and MacVicar, 1996) of muscarinic stimulation and following a single action potential or a burst, respectively.

and with a series of action potentials of decreasing frequency upon sustained depolarization (spike frequency adaptation, SFA; Fig. 1 B). A small fraction of these cells exhibits endogenous bursting activity, which consists of a cluster of closely spaced spikes riding on a large depolarizing envelope (Kandel and Spencer 1961; Wong and Prince, 1981; Jensen et al., 1994). Intermediate firing patterns amongst CA1 neurons have also been identified, suggesting that this process is highly dynamic and depends on a multiplicity of factors, such as the
state of intrinsic ionic mechanisms (density and functional properties of ion channels, pumps and transporters in the different neuronal compartments) and changes in the immediate environment of the cell (Jensen et al., 1994; Jensen and Yaari, 1997; Azouz et al., 1997; Su et al., 2001). This plasticity of firing modes might also be relevant in pathophysiological conditions involving abnormal neuronal excitability such as epilepsy, Alzheimer’s disease, chronic stress (Beck and Yaari, 2008).

1.1 Afterpotentials
Intrinsic excitability results from the dynamic spatial and temporal relationship between the active ionic conductances triggered in the cell upon arrival of a stimulus and the passive properties of the cell membrane. Some of these conductances underlie so-called afterpotentials, which are post-spike events of variable duration that shift the membrane potential in the positive or negative direction and therefore constitute an endogenous modulatory mechanism for neuronal firing. Afterhyperpolarizations (AHP) and afterdepolarizations (ADP) are not mutually exclusive, as they can occur simultaneously and interact with one another, giving rise, under different conditions, to multiple patterns of activity in a single neuron, e.g. adaptation, plateau potentials, regular or burst firing (McCormick, 1999). Many afterpotentials have been described in pyramidal neurons of the hippocampus, triggered by diverse mechanisms and with different kinetics (Kandel and Spencer, 1961; Caeser et al., 1993; Congar et al., 1997; Storm, 1990; Stocker et al., 2004), but their physiological relevance and the identity of the molecular mechanisms mediating some of these events are still largely unresolved. The main obstacle in this regard has been the lack of appropriate pharmacological tools to study the underlying currents in isolation, as well as their complex and possibly overlapping mechanisms of
regulation by neurotransmitters.

1.1.1 Afterhyperpolarizations

Three kinetically distinct hyperpolarizing potentials have been described in the mammalian hippocampus: fast (f), medium (m) and slow (s) afterhyperpolarizations (Fig. 1 C). These follow single or bursts of action potentials, thereby limiting or slowing down the firing activity of neurons after sustained stimulation. Potassium channels that activate in response to changes in intracellular Ca\(^{2+}\) brought about by firing activity are important contributors to these potentials, but voltage-gated conductances that are slowly activating or long lasting also participate (Storm, 1990). The fAHP is mediated by a Ca\(^{2+}\)-and voltage-activated K\(^+\) current generated by the opening of large-conductance channels of the BK type (I\(_C\)). This current, sensitive to submillimolar concentrations of tetraethylammonium (TEA), is rapidly activated (~1 ms) by depolarizations beyond -40 mV and micromolar concentrations of intracellular Ca\(^{2+}\) and decays within a few milliseconds (Storm, 1990). In this manner, I\(_C\) allows repolarization of the action potential and produces a fast undershooting phase in membrane polarity (Lancaster and Nicoll, 1987; Storm 1987). In apparent opposition to these inhibitory actions, partial block of BK channels reduces the ability of the neuron to generate high frequency discharges at high stimulation (Gu et al., 2007), presumably because a reduction in BK-mediated repolarization prolongs the refractory period of the membrane by hindering the recovery of Na\(^+\) channels from inactivation. These observations reveal an additional facilitatory role of I\(_C\) in hippocampal neurons.

The mAHP lasts several hundreds of milliseconds and, at hyperpolarized voltages, its decay is shaped by the activation of the Cs\(^+\)-sensitive, mixed
cationic current $I_h$ (Storm, 1989), mediated by HCN channels activated by membrane hyperpolarization and modulated by cyclic nucleotides. The mAHP arises from the combined activity of Ca$^{2+}$-activated small conductance (SK; $I_{AHP}$) and voltage-gated (KCNQ; $I_M$) K$^+$ channels (Storm, 1989; Wang et al., 1998; Tzingounis and Nicoll, 2008; Stocker et al., 1999), although a small, BK-dependent component has also been suggested to influence this afterpotential (Lancaster and Nicoll, 1987; Storm 1989). SK channels are highly sensitive to intracellular Ca$^{2+}$ ($EC_{50}= 300$-700 nM), a feature ascribed to the constitutive tethering of the Ca$^{2+}$-sensing molecule calmodulin (CaM) to the C-terminus of SK channel subunits (Stocker, 2004). KCNQ channels, on the other hand, are gated by depolarizations beyond -60 mV and do not inactivate (Storm, 1990). Intracellular Ca$^{2+}$ has been shown to negatively modulate KCNQ subunits through interaction with CaM (Haitin and Attali, 2008). SK- and KCNQ-mediated components can be readily discerned using selective blockers, such as the bee venom toxin apamin or d-Tubocurarine (dTC) in the case of SK channels (Stocker et al., 1999), and XE991 in the case of KCNQ (Wang et al., 1998). By influencing the level of membrane repolarization during interspike intervals, the medium duration AHP helps set the spike frequency during the first 300 ms of sustained stimulation in CA1 neurons (early SFA; Stocker et al., 1999; Oh et al., 2000; Pedarzani et al., 2005; Shah et al., 2006). In contrast, some studies have failed to identify an apamin-sensitive AHP upon somatic depolarization despite the recognized distribution of SK subunits throughout the cellular compartments (Bowden et al., 2001; Lin et al., 2008), suggesting that it is not involved in the regulation of excitability (Gu et al., 2005); the reason for this discrepancy is currently not known.
Lastly, the slow afterhyperpolarizing current (slAHP) underlies the sAHP and has the most profound impact on the late phase of SFA, because it remains active for several seconds. This current is evoked by intracellular Ca\textsuperscript{2+} rises during action potentials or by Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (Lancaster and Adams, 1986; Shah and Haylett, 2000). The slAHP activates within 200-500 ms and decays slowly with a time constant > 1 s (Lancaster and Zucker, 1994; Sah and Clements, 1999), its kinetics being strongly dependent on temperature (Lancaster and Adams, 1986). It is not affected by apamin or by wide spectrum K\textsuperscript+ channel blockers such as tetraethylammonium (TEA) and 4-aminopyridine. Therefore, the molecular identity of the channels involved in the generation of the slAHP has been subject of great speculation. Recent studies in mice lacking KCNQ channels of the 2, 3 or 5 subtypes have implicated these voltage-sensitive subunits in the slow activation and decay of the slAHP in CA3 neurons (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010). However, the slAHP is not voltage-dependent and requires Ca\textsuperscript{2+} for its delayed activation, as demonstrated using Ca\textsuperscript{2+} chelators, Cd\textsuperscript{2+} and photolytic manipulations; these notions oppose the involvement of KCNQ channels. Another study by the same group presents evidence linking the neuronal Ca\textsuperscript{2+} sensor hippocalcalcin and the slAHP in CA3 neurons (Tzingounis et al., 2007). Notably, in the absence of hippocalcalcin, slAHP channel activation is strongly reduced, but not fully abolished. The persistence of a small sAHP component in hippocalcalcin knockout animals partly conciliates the above contradictions, suggesting that the current may be mediated by at least two different types of channels. Yet, the identity of the downstream targets of hippocalcalcin is still under consideration, as is the mechanism through which KCNQ channels can mediate two kinetically distinct conductances.
1.1.2 AHP modulation by neurotransmitters

The regulation of AHPs by transmitter substances was documented from early on. In particular, this seems to be a hallmark of the sI AHP, upon which converge several different neuromodulatory pathways, including noradrenergic, serotonergic, histaminergic and dopaminergic afferents (Stocker et al., 2004). Interneuron-derived substances such as corticotropin releasing factor, vasoactive intestinal peptide and calcitonin gene related peptide, can also influence the sI AHP (Aldenhoff et al., 1983; Haas and Gahwiler, 1992; Haug and Storm, 2000). All of these modulators strongly suppress the current in a cAMP and PKA dependent manner. In addition, the sI AHP is inhibited by metabotropic receptor-mediated signaling following glutamate or acetylcholine release, through a mechanism that does not involve PKA but, at least in part, the Ca\(^{2+}\) dependent kinase CaMK (Stocker et al., 2004). In comparison, neuromodulation of the fast and medium AHPs has been less extensively studied. The fAHP appears to be insensitive to noradrenaline and muscarinic agonists (Storm, 1990), whilst the I\(_M\) component of the mAHP is suppressed by muscarinic, glutamatergic and histaminergic stimulation and various peptides in many neurons (Brown and Passmore, 2009). The regulation of endogenous SK channels by neurotransmitters has not been investigated in detail, but there is evidence that glutamate and PKA may depress the apamin sensitive mAHP in neocortical and hippocampal pyramidal neurons respectively (Pedarzani and Stocker, 2008).

By enhancing excitability, these mechanisms, which are coupled to the performance of particular tasks or to the regulation of the state of awareness of the brain, ultimately modulate the integration and output of the pyramidal neuron. Importantly, although in many cases masked by the AHPs, the
existence of depolarizing afterpotentials and their participation in the amplification of the effects of these transmitters should not be disregarded.

1.1.3 Afterdepolarizations

The existence of ADPs and their involvement in the hyperexcitability of pyramidal neurons has been known for many years (Kandel and Spencer, 1961; Fujita, 1975; Wong and Prince, 1981; Knöpfel et al., 1990); however, for several reasons they have remained relatively elusive in comparison to AHPs. ADP currents are thought to underlie burst activity and plateau potentials in the hippocampus and other regions of the brain. They are therefore important for signal integration and storage as they facilitate neurotransmitter release, increasing the reliability of synapses (Lisman, 1997) and may also play an important role in the generation of ictal depolarizations in central neurons (McCormick and Contreras, 2001). Two distinct types of ADP currents have been observed so far in cortical and hippocampal neurons: fast or spike $I_{ADP}$ (f$I_{ADP}$) and slow $I_{ADP}$ (s$I_{ADP}$) (Fig. 1D). These ADPs are temporally superimposed with AHPs, which means that they have the potential to counteract the dampening of excitability induced by post-spike hyperpolarizations, in this way adding to the multiple pathways that converge in their modulation. This thesis project centres upon a novel medium duration Ca$^{2+}$-dependant ADP current ($I_{ADP}$) characterized in CA1 neurons of rat hippocampal slices (Giovannini, Costello, Whale, Stocker and Pedarzani, unpublished observations). The known physical, pharmacological and physiological features of all three ADP currents are described below.

1.1.4 Fast and slow ADPs

Pyramidal neurons of the hippocampus are capable of generating single spikes
or bursts. Each single spike is followed by a clearly defined fast ADP (also called spike ADP), which delays the repolarizing effects of the fAHP in these neurons. Bursts are clusters of action potentials thought to be generated by the summation of single spike ADPs (Kandel and Spencer, 1961; Fujita, 1975). A number of studies have revealed that in fact fADPs differ between single- and burst-firing pyramidal neurons, pointing at the possibility that they may result from the activation of different conductances (Jensen et al. 1996; Metz, 2005). In particular, intrinsic bursters display an ADP that appears like a depolarizing shoulder on the repolarizing phase of the action potential, which has been attributed to the activation of slow voltage-gated channels. This contrasts with the passive decay of the ADP in non-bursting neurons. Despite these variations, the overall profile of the spike ADP is that of a 10-20 mV depolarization which decays with a time constant in the order of tens of milliseconds (Wong and Prince, 1981; Jensen et al., 1996; Metz, 2005). Amongst the proposed conductances underlying the spike ADP(s) in the hippocampus are the persistent Na⁺- (Jensen et al., 1996; Azouz et al., 1996; Su et al., 2001; Yue et al., 2005) and R-type Ca²⁺ (Metz et al., 2005) currents. Fast ADPs have also been described in several areas of the cortex, where they are mediated by Ca²⁺- and voltage- dependent currents, have slightly slower kinetics and are possibly generated by entirely different conductances (Connors and Gutnick, 1990; Foehring and Waters, 1991; Friedman et al., 1992; Haj-Dahmane and Andrade, 1997). T-type Ca²⁺ currents (Foehring and Waters, 1991), electrogenic Na⁺/Ca²⁺ exchange (Friedman et al., 1992) and Ca²⁺-activated non-selective cation currents (CAN channels; Haj-Dahmane and Andrade, 1997) are some of the nominated cellular mechanisms underlying cortical ADPs.
The interaction between spike ADP and kinetically matching voltage-gated K\(^+\) currents has been studied in some detail (Yue and Yaari, 2004; Metz et al., 2007). Thus, pharmacological modulation of I\(_M\) (but not the SK mediated I\(_{AHP}\)) with selective concentrations of linopiridine or XE991 strongly enhances the spike ADP and promotes bursting in CA1 neurons, suggesting that, under normal conditions, this K\(^+\) current prevents excessive depolarization of the membrane caused by persistent Na\(^+\) channels. This has been demonstrated to occur in and proximal to the soma of the pyramidal neuron (Yue and Yaari, 2004; Yue et al., 2005). In the dendrites R-type Ca\(^{2+}\) and D-type K\(^+\) channels seem to participate in a similar functional interplay (Metz et al., 2007).

In CA1 neurons, an additional, more prolonged I\(_{ADP}\) is induced upon stimulation of muscarinic or metabotropic glutamate receptors (mGluRs) (Crépel et al., 1994; Congar et al., 1997; Fraser and MacVicar, 1996). The neurotransmitter-induced I\(_{ADP}\), which can last several seconds, underlies plateau potentials, possibly the cellular basis of the sustained depolarization observed during the ictal phase of seizures (Fraser and MacVicar, 1996). It has been proposed to be mediated by cyclic nucleotide-gated channels in the case of the muscarinic-dependent ADP (Kuzmiski and MacVicar, 2001) or CAN channels for the mGluR-dependent ADP subtype (Crépel et al., 1994; Congar et al., 1997). A similar current has been reported in CA3 pyramidal neurons (Caeser et al., 1993; Guérineau et al., 1995; Gee et al., 2003) and in layer V neurons in the prefrontal cortex (Haj-Dahmane and Andrade 1996; 1998; 1999), where it results from the activation of a transient receptor potential conductance (Yan et al., 2009). Slow ADP currents have a nearly linear relationship with voltage, are not temperature dependent and are induced by raised intracellular Ca\(^{2+}\), although the different sensitivity to extracellular Cd\(^{2+}\) of mGluR- and muscarinic-
induced sADPs in CA1 neurons advocates that the sources of this Ca$^{2+}$ might be diverse (Crépel et al., 1994; Fraser and MacVicar, 1996). Nevertheless, the inhibition of the sIADP in the presence of high concentrations of BAPTA is a constant, as is the requirement for receptor-triggered second messenger pathways for its activation. Accordingly, the current cannot be induced by suppression of slow outward K$^+$ currents by other neuromodulators such as noradrenaline (Caeser et al., 1993), although without specific blockers of the sAHP or sADP this remains a controversial issue.

1.1.5 Ca$^{2+}$-dependant IADP in hippocampal pyramidal neurons

In rat hippocampal slices, depolarizing voltage-clamp steps delivered in the presence of tetrodotoxin (TTX) and moderate concentrations of tetraethylammonium (TEA, 1-5 mM) induce a large influx of Ca$^{2+}$, which in turn, activates multiple secondary ionic and metabolic processes. Amongst these are the already described AHP currents, which are independently capable of maintaining a hyperpolarized membrane potential for variable periods. The IADP under consideration in the present study is a cationic current revealed by the pharmacological suppression of the IAHP, IM and the sIAHP. The specific SK channel blockers apamin, dequalinium and dTC are sufficient and equally efficient at unmasking this conductance. Blocking the M-current with XE991 or inhibiting the sIAHP with the cAMP analogue 8-CPTcAMP (Pedrazani and Storm, 1993) alone do not unmask IADP, but play a role in enhancing and sculpting this current in the presence of all other blockers (Giovannini et al., unpublished observations). IADP is triggered by Ca$^{2+}$ entering the cell through VGCC and decays with a time constant in the order of hundreds of milliseconds. As a result, IADP is completely abolished in the presence of extracellular Cd$^{2+}$ and by enhancing the buffering capacity of the neurons by intracellular application of a
Ca^{2+} chelator (BAPTA). One of the most distinctive features of I_{ADP} is its regulation by moderate heat, with a temperature coefficient higher than most ion channels and transporters ($Q_{10(23-25^\circ C)} = 19.5; Q_{10(26-30^\circ C)} = 7.5$). Additionally, in analogy with the f_{ADP} in these same neurons (Azouz et al., 1997), this current is sensitive to osmotic changes, its amplitude being increased by hypotonic extracellular solutions (Giovannini et al., unpublished observations).

An afterdepolarization such as I_{ADP} might generate has been described in nigral dopamine neurons upon blockade of SK channels with apamin (Ping and Shepard, 1999), where it was linked to the occurrence of regenerative plateau potentials. It was suggested that dihydropiridined-sensitive Ca^{2+} channels mediate this ADP because it could be blocked by nifedipine and showed a distinctive voltage-dependency. More recently, Park et al. (2010) identified an mGluR-induced ADP lasting over 200 ms in CA1 neurons (mADP). The authors propose that this conductance results from the upregulation of Ni^{2+}-sensitive R-type Ca^{2+} channels, which, as described, had also been implicated in the generation of the f_{ADP} by the same group. These findings, together with the fact that the mADP is greatly reduced by TTX, suggest that under glutamatergic and cholinergic stimulation, the boosting of the R-type Ca^{2+} (Tai et al., 2006) and persistent Na^{+} components of the spike ADP might give rise to a longer-lasting depolarization.

However, the Ca^{2+}-dependent I_{ADP} described by Giovannini et al. and examined in the present study corresponds to a previously unreported conductance for hippocampal pyramidal neurons, which does not require the engagement of receptor-induced second messenger pathways for its activation. The multifactorial nature of I_{ADP}, on the other hand, may reflect the activity of one or
more ion channels or transporters capable of counteracting the effects of AHPs under physiological conditions. This interplay between inward and outward conductances is likely to be targeted by neuromodulators, which would alter the amplitude and spatio-temporal features of the resulting afterpotential. Preliminary results show that $I_{\text{ADP}}$ may indeed participate in this dynamic interaction as implied by the sustained depolarizations, burst firing and reduction of action potential amplitude observed in the presence of fAHP (TEA), mAHP (dTC and XE991) and sAHP (8CPTcAMP) inhibitors at room temperature and at 30°C (Giovannini et al., unpublished observations). However, these results may quite easily lead to the assumption that it is the absence of K⁺ currents (voltage- and Ca²⁺-gated) that have such a profound effect in the firing properties of CA1 neurons, and are thereby not conclusive as for the specific role of $I_{\text{ADP}}$. The identification of the molecular identity of $I_{\text{ADP}}$ and its suppression by genetic manipulations in neurons will ultimately enable the dissection of the relative contributions of these inter-related conductances to neuronal function.

1.2 Clues to the molecular identity of $I_{\text{ADP}}$

Considerable effort has been granted to the characterization of the pharmacological profile of many ionic conductances, and the lack of specific, clear-cut pharmacological tools is often a major set back in electrophysiology. Neurons are complex systems where it is almost impossible to record single ionic currents in isolation and this has been the main obstacle in the pharmacological description of $I_{\text{ADP}}$.

The possibility that a Ca²⁺ channel may mediate $I_{\text{ADP}}$ seems unlikely, given that this current disappears in the presence of intracellular BAPTA, as expected for
a Ca$^{2+}$-activated channel rather than a voltage-gated Ca$^{2+}$ channel (Giovannini et al., unpublished observations). Equally, previous work has explored the possibility that $I_{\text{ADP}}$ be mediated by the Na$^+$/Ca$^{2+}$ exchanger present in CA1 neurons, but neither the widely used inhibitor benzamil (up to 250 μM), nor more specific drugs like KB-R7943 (100 μM) and XIP (10 μM) had any appreciable effect on the current (Giovannini et al., unpublished observations).

Na$^+$/Ca$^{2+}$ and Na$^+$/H$^+$ exchangers, acid-sensing cation channels, cyclic nucleotide-gated channels and a subset of transient receptor potential (TRP) channels are all targeted at low, medium or higher concentrations by the compound amiloride, but when applied in the slice preparation at 100 μM amiloride did not reduce $I_{\text{ADP}}$ (Giovannini et al., unpublished observations).

Given the cationic nature of $I_{\text{ADP}}$ and its steep sensitivity to temperature in the physiological range, the strongest contenders at present are TRP channels, a large family of cation permeable channels originally discovered in *Drosophila* photoreceptors, where they mediate prolonged depolarizing potentials in response to continuous light (Minke, 1977; Montell et al., 1985). These channels are almost ubiquitously expressed and display polymodal activation (including temperature-, and Ca$^{2+}$-, in some cases) and complex pharmacological profiles. Their main properties are reviewed here.

### 1.3 Overview of transient receptor potential channels

TRP channels have been classified according to their sequence similarity into seven different subfamilies, only six of which are represented in mammals: canonical (TRPC), vanilloid receptor (TRPV), melastatin (TRPM), mucolipins (TRPML), ankirin transmembrane protein (TRPA) and polycystins (TRPP); the seventh subfamily NompC-like or TRPN comprises proteins in tunicates, flies
and worms (Clapham et al., 2005; Alexander et al., 2008; Wu et al., 2010; Fig. 2). These subfamilies are only loosely related to each other (~20 % overall sequence identity), but the degree of amino acid sequence similarity within each group reaches up to more than 90 % in some cases. TRP channels are connected by structural similarities and selectivity criteria. Although they are not sharply dependent on trans-membrane voltage changes like classical VGCC, their opening in response to a wide variety of physical and chemical stimuli enables them to function as highly specialized transducers allowing the non-selective influx of cations, thereby driving membrane depolarization and initiating Ca\textsuperscript{2+} signaling in many cells (Clapham et al., 2001).
1.3.1 Molecular features

All 28 *trp* gene products have been predicted to form six transmembrane domain (TM1-TM6) polypeptides with intracellular N- and C- termini. They assemble as tetramers into cation permeable channels (Ramsey *et al.*, 2006; Latorre *et al.*, 2009), with a pore-forming loop located between TM5 and TM6 (Owsianik, *et al.* 2006). Therefore, TRP channels are architecturally, but not functionally, similar to voltage-gated K$^+$ channels, since the positive residues that contribute to voltage sensitivity are absent or scarce in the S4 helix of TRPs. The overall structure and tetrameric assembly of TRP subunits has been substantiated by low-resolution three-dimensional reconstruction from electron microscopy images for TRPC3 (Mio *et al.*, 2008), TRPM2 (Maruyama *et al.*, 2007) and TRPV1 channels (Moiseenkova and Wensel, 2009).

Additionally, TRP channels can exist as homomers or heteromers, and channel properties can vary quite substantially upon heteromeric assembly (Cheng *et al.*, 2007; Chung *et al.*, 2008), an issue not completely understood in native tissues. A fascinating and unique feature of TRP channels is the ability of subunits from different subfamilies to associate with one another, although, so far, this has only been described to occur between TRPP and TRPC/TRPV subunits in expression systems (Köttgen *et al.*, 2005; Bai *et al.*, 2008). Nevertheless, these findings complicate the characterization of native TRP-mediated events, because the hybridization of cross-family phenotypes makes it even more difficult to manipulate the resulting currents with known pharmacological tools.

Other common molecular features of TRP proteins include coiled coil regions within the N- and C-terminal cytoplasmic domains of TRPCs, TRPVs, TRPMs
and TRPP but not TRPAs or TRPMLs. These have been demonstrated to participate in the heteromerization of TRPV, TRPM and TRPP channels (García-Sanz et al., 2004; Mei et al., 2006; Bai et al., 2008). Indeed, mutating the amino acid sequence in the coiled coil domains of several TRPs has proved useful in the generation of dominant negative subunits, which prevent normal channel assembly and trafficking. The amino-terminal region of TRPA, V and C channels harbors large stretches of ankyrin repeats that also serve as domains of interaction with other proteins, including subunits in the multimeric assembly (Latorre et al., 2009). In addition, the polypeptidic chains of several members of this superfamily contain a highly conserved block of ~25 residues forming the so-called TRP domain with a ‘TRP box’ (WKFQR) located closely after TM6 in the intracellular C-terminal segment of TRPC, V and M (Latorre et al., 2009). The TRP motif does not resemble any other amino-acid sequence of known function, and has been implicated in heteromerization and ‘negative allosteric modulation’ of channel gating (Garcia-Sanz et al., 2007; Rohacs and Nilius, 2007). In keeping with this role, peptides patterned on the TRP domain template to physically interfere with the conformational changes required in this region for TRPV1 gating inhibit channel function (Valente et al., 2011). The high level of selectivity of these novel competitive antagonists is of great interest for the identification of TRP-mediated events and will surely be made extensive to other TRP subunits.

1.3.2 Selectivity

Most TRP channels are mixed cationic, discriminating poorly amongst the different cations ($P_{Ca/Na}<10$), the only exceptions being TRPM 4, 5 and TRPM3α1 ($P_{Ca/Na}<0.05$) which are exclusively permeable to monovalents, and the $Ca^{2+}$-selective TRPV5, 6, and TRPM3α2 ($P_{Ca/Na}>100$) (Flockerzi, 2007; Wu...
et al., 2010). TRP channels do not exhibit a clear consensus sequence in their predicted selectivity filter, such as the GYGD signature motif present in all K⁺ channels (Owsianik et al., 2006). Furthermore, by contrast to most ion channels where permeation properties are considered to be an invariable and defining characteristic, the selectivity of several TRP channels seems to be a rather plastic feature. Initially, some TRP channels appeared to have large pore diameters, which allowed them to permeate organic cations such as NMDG⁺, spermine or TEA. In recent years, several groups have been able to demonstrate that TRPV1, 3, 5 and TRPA1, but not TRPM8, actually experience changes in the physical dimensions of their pore after persistent stimulation thereby being able to change regular permeation patterns (Latorre et al., 2009). Dynamic changes in pore size may depend on factors as diverse as agonist binding, pH or temperature. To further complicate selectivity matters, it should be noted that most TRP channels have one or more splice variants (Ramsey et al., 2006). In the case of TRPM3, alternative splicing gives rise to ion channels with markedly different abilities to discriminate between cations, ranging from highly monovalent selective to being almost exclusively Ca²⁺ permeant (Oberwinkler and Philipp, 2007).

1.3.3 Regulation of channel activity

TRP channels can be activated by many diverse cellular signals including voltage, stretch, temperature, second messengers (such as PIP₂, PLC, DAG), Ca²⁺, ADP ribose, ATP, PKC, PKA, etc. In fact, the majority of TRP channels are thought to detect the coincidence of different stimuli. Typically, canonical TRP channels are coupled to receptor-activated intracellular cascades and store depletion, whilst members of the vanilloid, melastatin and ankyrin transmembrane subfamilies are notorious for their ability to integrate a variety of
chemical (exogenous or metabolically-derived molecules) and thermal stimuli. TRPP channels most likely transduce mechanical changes in the membrane, and TRPMLs are located in endolysosomal compartments where they sense intra-organelle pH fluctuations and mediate Ca\(^{2+}\) release (Wu et al., 2010).

Of particular relevance to this study is the direct or indirect relationship that many TRP channels have with Ca\(^{2+}\), in some cases like TRPC3, (Zitt et al., 1997), TRPM2 (Du et al., 2009), TRPM4 (Launay et al., 2002), TRPM5 (Prawitt et al., 2003) and TRPA1 (Doerner et al., 2007) even being directly gated by this second messenger. In other instances, the interaction with Ca\(^{2+}\) appears to be modulatory. Accordingly, many members of the superfamily exhibit (often multiple) CaM or Ca\(^{2+}\) binding EF domains in their cytosolic N- or C- termini (Zhu, 2005) (Fig. 2). Negative modulation of TRP channels by Ca\(^{2+}\) appears to be intimately connected with PLC-mediated PIP\(_2\) depletion. In analogy with many ion channels, this membrane phospholipid has been demonstrated to exert a strong modulatory effect on most TRP channels (Rohacs, 2009).

The other outstanding feature of some TRP channels is their temperature sensitivity. ThermoTRPs comprise a subset of (so far) nine representatives from the TRPV, TRPA and TRPM subfamilies (Talavera et al., 2005; Dhaka et al., 2006; Huang et al., 2006; Togashi et al., 2006), mainly expressed in sensory systems, where they contribute to the organism’s response to environmental temperature changes and mediate the thermal sensations evoked by many naturally occurring compounds. Curiously, temperature sensing TRP channels also exist in other tissues such as heart, spleen, liver, pancreas, brain (just to mention a few), where they are not exposed to large temperature fluctuations. These TRP channels are usually responsive to physiological temperature
ranges and are therefore proposed to be constitutively active (Güler et al., 2002; Smith et al., 2002; Xu et al., 2002; Togashi et al., 2006; Shibasaki et al., 2007). Their specific roles within those tissues, however, are less clearly defined.

The physical nature of the TRP temperature sensor is a much-debated subject. This issue has been tackled with the use of molecular chimeras and site directed mutagenesis. The prevalent views are that the thermo-sensing mechanism is either linked to shifts in the shallow voltage activation curves of some TRPs or represents an allosteric unit physically coupled to a voltage- and temperature-independent channel gate (Voets et al., 2004; Latorre et al., 2009). Overall, there is a consensus regarding the interaction of voltage, agonists and temperature during channel gating, a process studied in great detail for TRPV1 and TRPM8, where temperature activation has been linked to a region in the C-terminus (Brauchi et al., 2006). The thermosensing characteristics of TRP channels are specifically important for this work given the temperature dependence exhibited by $\text{I}_{\text{ADP}}$.

1.3.4 TRP channels in the hippocampus

TRP proteins have been detected in virtually every cell type in the body. In many cases, different TRP family members coexist within one cell, in the same or separate compartments. TRP channels have been historically implicated in sensory processes because they activate in response to a huge variety of environmental cues (Damann et al., 2008; Chang et al., 2010; Koike et al., 2010; Wu et al., 2010), but a novel role for this family of ion channels in the central nervous system is beginning to emerge (Moran et al., 2004; Talavera et al., 2008a; Sharif-Naeini et al., 2008).
Hippocampal neurons express members of the TRPC, TRPV, TRPM and TRPP subfamilies (Fig. 3). Functional identification of endogenous homomeric and heteromeric TRP assemblies has been hampered by the lack of specific blockers and poor selectivity of the available pharmacological tools, forcing reliance on siRNA and other genetic ablation strategies. However, significant evidence has been gathered in support of TRP channel involvement in hippocampal neuron function.

TRPC channels have been implicated in neurite growth, pathfinding and spine formation signaled by growth factors (Greka et al., 2003; Bezzerrides et al., 2004; Amaral and Pozzo-Miller, 2007; Davare et al., 2009). Functional expression of TRPC 3 and 5 in the newly formed growth cones and neurites is dynamically regulated through a process of fast vesicular packaging, translocation and insertion, which allows tight spatio-temporal control of growth factor induced signals. In CA3 neurons, canonical TRPs are also proposed to underlie the slow depolarizing currents evoked by group I mGluR activation (Gee et al., 2003). Using GFP-tagged constructs in organotypic slices, Yan et al. (2009) recently demonstrated that the analogous sADP induced by muscarinic agonists in cortical pyramidal neurons is facilitated by overexpression of TRPC5 and 6 and suppressed by non-conducting TRPC5 mutants. Meanwhile, studies with mice lacking the capsaicin receptor TRPV1 have reported impaired synaptic plasticity and hippocampal dependent learning in these animals (Marsch et al., 2007). Likewise, heat-stimulated cytosolic Ca\(^{2+}\) rises and patch clamp measurement of whole-cell currents in neurons cultured from the hippocampus of wild type and TRPV4 null mice reveal that these channels participate in the regulation of the resting membrane potential and firing threshold at physiological temperatures (Shibasaki et al., 2007). On the
<table>
<thead>
<tr>
<th>TRP</th>
<th>Hippocampus/brain detection</th>
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<th>Inhibitors</th>
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Figure 3. Pharmacology of hippocampal TRP channels. Abbreviations: RT-PCR (reverse transcription polymerase chain reaction), ISH (in situ hybridisation), NB (Northern blot), WB (Western blot), IF (immunofluorescence), Ephys (electrophysiology); (*) experiments performed in H19-7 hippocampal neurons. Highlighted in bold are the most widely used TRP modulators.
other hand, members of the TRPM subfamily seem to be mainly involved in neuronal death following an ischemic insult. Oxidative stress-sensitive TRPM2 and 7 are important Ca\(^{2+}\) influx pathways under these conditions (Lipski et al., 2006; Wei et al., 2007; Olah et al., 2009; Sun et al., 2009; Bai and Lipski, 2010).

1.4 Summary

In summary, pyramidal neurons are endowed with the intrinsic ability to regulate their spike output in a fast and dynamic manner. The existence of afterpotentials is one of such intrinsic mechanisms. ADP currents have been described elsewhere for the hippocampus and cortex; their biophysical features, however, diverge from the Ca\(^{2+}\)-dependent \(I_{\text{ADP}}\) described by Giovannini et al. in CA1 neurons. Previously reported ADP currents present alternative activation mechanisms and kinetics, possibly due to different underlying molecular mechanisms. TRP channels represent a vast group of proteins abundant in many tissues including the hippocampus, exhibiting features similar to those of \(I_{\text{ADP}}\), namely: i) they are generally non-selective cation channels; ii) present weak voltage sensitivity; iii) a subset of TRP channels have \(Q_{10}\) values up to three fold greater than those reported for most ion channels and transporters and iv) many show direct or indirect sensitivity to [Ca\(^{2+}\)]\(_{\text{in}}\).

The overall aim of this PhD project was the molecular identification of the channel/s underlying \(I_{\text{ADP}}\) and their function in hippocampal neurons. The first part of the thesis deals with the characterization of \(I_{\text{ADP}}\) in primary cultures of hippocampal pyramidal neurons, an ideal system for pharmacological and genetic interventions. An extensive pharmacological characterization of \(I_{\text{ADP}}\) in this preparation is also presented, which exposes the involvement of TRP channels in the generation of this Ca\(^{2+}\)-activated conductance. Finally, the
selective modulation of $I_{ADP}$ in hippocampal slices was found to have a significant impact on pyramidal neuron excitability.
Chapter Two

Materials and Methods
2.1 Materials

2.1.1 Consumables

4 and 6 well plates: Nunc
Coverslips: Ø 10 mm, 22 x 22 mm Menzel-Gläzer, BDH
Filters: syringe filter (0.22 µm), stericup (0.22 µm), Millex-Millipore, Whatman
Glass capillaries: borosilicate, Slice electrophysiology Hilgenberg, Kimble
(1.5 x 0.3 mm), cell culture electrophysiology
(1.5 x 1.8 mm)
Microcentrifuge tubes: 0.5, 1.5, 2 ml Sarstedt
Glass "Pasteur" pipette VWR
PCR reaction tubes: 0.2 ml Applied Biosystems
Petri dishes: Ø 35 mm, Ø 55 mm, Ø 9 cm Nunc
Plastic transfer pipette Sarstedt
Plastic tubes: 10 ml, 15 ml, 50 ml Sarstedt
Screw cap tubes: 12 ml Greiner
Serological pipettes: 5 ml, 10 ml, 25 ml Sarstedt
Slides: 24 x 60 mm Menzel-Gläzer
Syringes: 1 ml, 2 ml, 5 ml, 10 ml Terumo
Tissue culture flask: T25 Nunc/Sarstedt

2.1.2 Equipment

Antivibration table Physic-Inst., TMC
Cameras: VW CU-204, C4742-95, MicroPublisher CCD Panasonic, Hamamatsu, Qimaging
Centrifuges: 5415-D and 5415-R, J2-MI, Megafuge 1.0R Eppendorf, Beckman, Heraeus
Electrophoresis cell: BioRad Mini-Protean II
Fluorescence Filters:
Axiohot: Set 15, Cy3 detection Zeiss
(Exc: BP 546-512, BS: FT580, Em: LP590)
Axiovert: Longpass emission filter (FITC/EGFP) Chroma
(Exc: HQ480/40, BS: DCLPQ505, Em: HQ510LP)
Leica: Leica L5 Leica
(Exc: BP480/40, BS: 505, Em: BP527/30)
Hood for dissection Envair
Hood for tissue culture
Incubators: cell culture (37°C, 5% CO₂),
Microelectrode pullers: PP830, L/M-3P-A, DMZ-Universal
Incubators: cell culture (37°C, 5% CO₂), Heraeus, Sanyo
Microelectrode pullers: PP830, L/M-3P-A, DMZ-Universal Narishigue, ListMedical
Zeitz Instrumente
Micromanipulators: LN SM1
Microscopes: Axioskop, Axiovert 200, Axiophot, DM IRB
Oscilloscope: TDS 320
Osmometer: Vapro®
Patch Clamp Amplifiers: EPC9, 10
PCR System: 2400
pH meters: 766, RL150
Pressure control unit: MPCU-3
Pump: Dymax 30
Scales: analytical, digital LA1205, digital PB3002
Sequencer: ABI Prism (3100-Avant)
Shaker: Innova 4230, 30-35°C, 5% CO₂
Spectrophotometer: NanoDrop
Temperature controller: TC-324B
Thermomixer
Tissue slicer: VT 1000S
UV lamp: short arc mercury lamp103W/2
UV trans-illuminator: 750-M UVL-24, (365 nm), UVP

2.1.3 Kits and chemicals

BigDye™ Terminator (v.1.1) Applied Biosystems
Lipofectamine™ 2000 Invitrogen
NucleoSpin Extract Machery-Nagel
NucleoSpin Plasmid Machery-Nagel
Nucleobond PC 100 Machery-Nagel
ProLong Antifade Molecular Probes

2APB Sigma
4αPhorbol Calbiochem
8CPTcAMP-Na Sigma
ACA Biomol
ADPR Sigma
Agar – bacto Difco
Agar – select
Agarose Ultra-Pure
AMP-Na
Ampicillin
APV
B27 supplement (50x)
BAPTA-K₄
Blasticidin
Bovine serum albumin
CaCl₂
cADPR
Dithiothreitol
DMEM-F₁₂
DMSO
dTubocurarine-Cl
EDTA
Ethanol
Ethidium Bromide (10 mg/ml)
Fetal calf serum
FFA
GdCl₃
Glucose
Glucose (tissue culture, 45%)
GTP-Na
H₂O (cell culture)
H₂O (molecular biology)
H₂O (intracellular solution)
HBSS (10x)
HEPES
HEPES (tissue culture, 1M)
Horse Serum
Industrial Methylated Spirits (IMS)
Isopropanol
KCl
Gluconic acid, potassium salt (KGluconate)
KH₂PO₄
L-Glutamine (200 mM)
LaCl₃
Gibco
Invitrogen
Sigma
Roche
Ascent Scientific
Invitrogen
Sigma
Invivogen
Sigma
Fluka
Biomol
Invitrogen
Sigma
Ascent Scientific
IBI technical
VWR, Fisher
Sigma
Sigma
Fluka
VWR, Fischer Scientific
Fluka
Baxter
Gibco
Romil
Invitrogen
Fluka
Invitrogen
Invitrogen
VWR, Fisher
Fischer Scientific
VWR, Sigma, BDH
Sigma
Merck
Invitrogen
Fluka
Luria Broth  
MEM  
MgCl₂  
NaCl  
NaHCO₃  
Na₂HPO₄  
NaOH  
NBQX  
Neurobasal  
NMDG  
OPTI-MEM  
Paraformaldehyde  
PBS (tissue culture, 1x)  
PDBu  
Penicillin/Streptomycin  
Poly-D-Lysine (MW 70 000-150 000)  
Pyruvic acid (200 mM)  
RuR  
SKF96365 hydrochloride  
Spermine  
TAE (50x)  
TEA  
Tetracycline  
Tris-Base  
Triton X-100  
Trypan blue  
Trypsin (hippocampal neurons, 2.5 %)  
Trypsin-EDTA (HEK cells, 0.05 %)  
TTX  
Tween-20  
XE991  
X-Gal  
ZD7288  
Zeocin  

2.1.4 Enzymes, Antibodies and Vectors

Alkaline Phosphatase  

Roche
KOD hot start DNA-polymerase (1 U/µl)    Novagen
Pfu DNA-polymerase (2.5 U/µl)     Stratagene
T4 DNA-Ligase (1 U/µl)     Roche

Anti-FLAG M2 monoclonal antibody    Sigma
Cy3-conjugated goat anti-mouse IgG (H+L)    Jackson

BlueScript KS⁺ (II)    Stratagene
pcDNA3    Invitrogen

Restriction enzymes were purchased from Roche, New England BioLabs and BDH

2.1.5 DNA markers

DNA Ladder 1 kb    Invitrogen
12216, 11198, 10180, 9162, 8144, 7126, 6108,
5090, 4072, 3054, 2036,1636, 1018, 506, 396,
344, 298, 220, 154, 134, 75

DNA Ladder 100 bp    Invitrogen
2072, 1500, 1400, 1300, 1200, 1100, 1000, 900,
800, 700, 600, 500, 400, 300, 200, 100

2.1.6 Oligonucleotides

T7    TAATACGACTCACTATAGGG
P2135    TAGGCCTGTACGGTGAGGTGCAGG
P1496    TAGAAGGCACAGTCGAGG
P2201    CCTCATCCACAACCAGCGCGCCGGTGGACTG
P2202    CAGTCCACCCGGCGCTGGTTGTGATGGAG
P2203    CCTGACGAGTGCGGGCTGATGA
P2204    CGAGTGCTACCGGAAGGACGAA
P2205    CTCTACCTGTCCATCAACACAT
P2206    CGAGTGCTACCCGGAAGGACGAA
P2207    CGGCGGAATTCAGTAGTGAGCCCCGAACTCAGCG
P2208    CAAGAAACAGCTGGAGAAGAACGA
T3    TAATACGACTCACTATAGGG

All primers were purchased from MWG
2.2 Methods

2.2.1 Cell culture and acute slice preparation

Coverslip sterilization and coating

For the purposes of cell culture 10 mm glass coverslips were first subjected to a 30 min wash in 100 % IMS followed by 3 rinses in 75 % IMS. Sterilization was achieved by baking at 200°C for 8-10 hours. The coverslips used for neuronal cultures were coated by immersion in a 100 µg/ml Poly-D-Lysine solution for 3-24 hours at 37°C. They were subsequently washed 3x with 1 ml deionized water and returned to the incubator until their use. In the case of immunocytochemistry experiments, 22 x 22 mm glass coverslips were sterilized in the same manner and briefly treated with the poly-D-lysine solution for 10 min at room temperature (RT, 21-24°C), after which time they were rinsed with water and stored under the hood until needed.

Primary culture of hippocampal neurons

All procedures strictly followed Home Office regulations under the Animal (Scientific Procedures) Act of 1986. P0 Sprague-Dawley rats were decapitated and both brain hemispheres quickly dissected out and transferred to a 35 mm dish containing cold (4°C) dissection medium (DM). The meninges were removed and the hippocampus dissected out and moved to a dish with fresh DM, where it was dissected into 7-8 small pieces before a 15 min enzymatic digestion with trypsin (70 µg/mL) at 37°C. After this time, the trypsin solution was removed by rinsing twice with DM and twice with attachment medium (AM). Mechanical dissociation of the tissue was achieved by pipetting up and down 5-10 times with standard fire-polished Pasteur pipettes of progressively smaller tip sizes. The undissociated tissue was left to settle at the bottom of the tube and
the cell suspension was centrifuged for 3 min at 200xg to eliminate residual enzyme solution. The pellet was re-suspended in AM, the suspension stained with 10 % trypan blue and the number of live cells determined. Typically this was in the range of 165 x 10^4 viable cells for every three pups. Cells were plated onto poly-D-lysine-coated glass coverslips at 45 000 cells/well (total volume 500 µl). The AM was replaced with growth medium 6-24 hours after plating and every 5 days half of the growth medium (GM) was replaced by fresh medium. The cells were kept for a maximum of 30 days in a humidified incubator at 37°C, 5 % CO₂. Day in vitro (DIV) 1 was designated as the day of culture preparation.

Hippocampal culture development was documented with bright field Leica Modulation Contrast photographs at each DIV using an inverted Leica DM IRB microscope equipped with a 40x objective and a C4742-95 Hamamatsu camera controlled by OpenLab software v5.02 (ImproVision, Perkin Elmer, USA).

<table>
<thead>
<tr>
<th>Dissection Medium (DM)</th>
<th>HBSS 1x</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HEPES 10 mM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Attachment Medium (AM)</th>
<th>MEM 1x</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Horse Serum 10 %</td>
</tr>
<tr>
<td></td>
<td>Glutamine 2mM</td>
</tr>
<tr>
<td></td>
<td>Pyruvic Acid 1 mM</td>
</tr>
<tr>
<td></td>
<td>Glucose 0.6 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Growth Medium (GM)</th>
<th>Neurobasal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glutamine 2mM</td>
</tr>
<tr>
<td></td>
<td>B27 supplement 2 %</td>
</tr>
<tr>
<td></td>
<td>Glucose 0.6 %</td>
</tr>
<tr>
<td></td>
<td>Pen/Strep 100 U/ml, 100 µg/ml</td>
</tr>
</tbody>
</table>

**Maintenance and splitting of cell lines**

**HEK 293 cells** - HEK 293 cells were purchased from the German Collection of Microorganisms and Cell Culture and allowed to grow in T25 flasks in the incubator at 37°C and 5 % CO₂ until confluency (80-90 %). The medium was
aspirated and dead cells and debris were removed by briefly washing with 4 ml PBS. Detachment and splitting of cells were performed by adding 1 ml of a 0.05 % Trypsin/EDTA solution. This volume was distributed evenly so as to cover the entire cell layer and the excess enzyme removed. Digestion proceeded for 30-60 sec at 37°C, after which 4 ml HEK complete medium was added and cells were dissociated mechanically. A fresh culture was generated using 500 µl of cell suspension in a total volume of 5 ml.

<table>
<thead>
<tr>
<th>HEK complete medium</th>
<th>DMEM/F12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fetal Bovine Serum 10%</td>
</tr>
<tr>
<td></td>
<td>L-Glutamine 2 mM</td>
</tr>
<tr>
<td></td>
<td>Pen/Strep 100 U/ml, 100 µg/ml</td>
</tr>
</tbody>
</table>

**Tetracycline-inducible FLAG-tagged hTRPM2 HEK cells (HEK-TRPM2)** - A cell line expressing the hTRPM2 channel with a FLAG epitope tag at its NH₂-terminus (HEK-TRPM2) was kindly provided by Dr. A Scharenberg (Seattle, USA). This stable cell line was generated using a tetracycline-regulated gene expression system driven by the (regulatory) pcDNA6 T/R and (inducible) pcDNA4 T/O vectors, which confer Zeocin and Blasticidin resistance to the host HEK cells. HEK-TRPM2 cells were grown in T25 culture flasks up to 90 % confluency in the presence of 0.2 mg/ml zeocin and 5 µg/ml blasticidin (37°C, 5 % CO₂). At this point the medium was aspirated and the cells rinsed with 4 ml PBS. They were detached from the culture flask by treatment with a 1 mM EDTA solution in PBS for 5 min at 37°C and gently dissociated. The suspension was centrifuged at 200xg for 1 minute and the supernatant aspirated. The cells were re-suspended in 3 ml HEK complete medium. A fresh culture was generated by seeding 500 µl cell suspension in 5 ml growth medium supplemented with antibiotics in a T25 culture flask. Expression of hTRPM2
channels in HEK-TRPM2 cells was induced by addition of 0.5 µg/ml tetracycline to the medium 16-24 hours prior to experiments.

**Preparation of dorsal hippocampal slices**

Transverse hippocampal slices (350 µm thick) were obtained from 21-25 day old male Sprague-Dawley rats. Animals were anesthetized by isofluorane inhalation and, after establishing that sufficient depth of anaesthesia was reached (i.e. absence of paw withdrawal reflex), they were decapitated and the skull removed leaving the brain exposed. Ice-cold artificial cerebro-spinal fluid (ACSF) was quickly poured over the head to cool the brain and slow down all metabolic processes. The brain stem and cerebellum were removed with sharp scalpel blades, the hemispheres separated with a single sagittal cut, and quickly dissected out with two frontal ~ 85° angle cuts with respect to the mid line. The hemispheres were securely glued (front side down) onto the stage of a Leica VT 1000S slicer. Slicing was performed in ice cold ACSF, bubbled with 95% O₂ and 5 % CO₂. Each section was transferred to a humidified surface chamber using the wide, polished end of a Pasteur pipette and allowed to recover for at least 1 hour before recording. The chamber atmosphere was at all times saturated with oxygen by means of continuous bubbling with carbogen. Sections obtained and maintained in this manner retained a good overall condition for 6-8 hours post dissection.

Dissection and maintenance ACSF

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>125 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>16 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>1 mM</td>
</tr>
<tr>
<td>pH 7.4 - 5</td>
<td>with CO₂</td>
</tr>
</tbody>
</table>
2.2.2 Immunocytochemistry in HEK-TRPM2 cells

HEK-TRPM2 cells were plated at a density of 75000 cells/well into a six well plate containing 22 x 22 mm poly-D-lysine coated glass coverslips. Twenty-four hours after induction the medium was aspirated and the coverslips were washed within the 6 well plates 3 times with 1x PBS. Cells were fixed with 2 ml of 4% Paraformadehyde/PBS per well for 10 min at RT. Subsequently, cells were rinsed twice with 2 ml 1x PBS, permeabilized in 0.2% Triton X-100 in PBS for 2 min and washed 4 times (5 minute/wash) with PBS. Coverslips were transferred to a humidified box and incubated in blocking buffer for 30 min at 37°C. Afterwards, the blocking buffer was replaced by the primary antibody anti-FLAG M2 (anti-FLAG M2 monoclonal, Sigma) diluted 1:200 in blocking buffer and the coverslips returned to the humidified box for 1 hour at 37°C. The cells were washed 3 times with 1x PBS (5 min/wash) and incubated with Cy3-conjugated goat anti-mouse antibody (Jackson) diluted 1:600 in blocking buffer, in a humidified box at 37°C for 30 min. Finally the cells were washed 3 times (5 min/wash) in 1x PBS before mounting using ProLong antifade. The mounting medium was allowed to harden at RT in the dark. The next morning the edges of the coverslip were sealed with transparent nail polish.

Images were acquired with QCapture software controlling a MicroPublisher CCD camera connected to an Axiophot inverted microscope with a 63x lens (1.25 NA). Fluorescent pictures were taken using a 103 Watt UV lamp and the Zeiss filter set #15 and processed with Adobe Photoshop CS.

10x PBS
H₂O
NaCl 1.3 M
Na₂HPO₄·x7H₂O 70 mM
NaH₂PO₄·xH₂O 30 mM
pH 7.3 adjust
autoclave
4% Paraformadehyde in PBS
1x PBS 100 ml
Paraformaldehyde 4g
pH to 7.3

Blocking solution
1x PBS
Bovine serum albumin 2.5%
Fetal calf serum 10%

2.2.3 Molecular biology

Restriction enzyme digestion of DNA

DNA was digested in 1.5 ml tubes in a total volume of 30-50 µl at the appropriate temperature in the presence of the restriction enzyme, 10x buffer (Roche A, B, L, M or H) and distilled water. In control digests 200-1000 ng DNA was used. For vector and fragment preparation the DNA amount was 1-2 µg. The digest was incubated for 1-2 hours and stopped by the addition of 5x DNA sample buffer.

5x DNA Sample Buffer
Ficoll 400 20%
EDTA 100 mM
Bromophenol Blue 0.25%
Xylene cyanol ff 0.25%

Phenol-chlorophorm extraction of DNA

One volume of phenol (pH 7.5) was added to the digest and vortexed. The phenol phase was removed after centrifugation (>10000xg) for 10 min and the water phase was extracted with 100 µl CHCl₃. After phase separation (centrifugation: >10000xg 5 min), the aqueous phase was transferred to a fresh 1.5 ml tube and the DNA was precipitated in the presence of 400 mM LiCl and 3 volumes EtOH (-80°C, 30 min). DNA was pelleted by centrifugation (15200xg, 15 min). The pellet was washed with 250 µl EtOH (75%) and pelleted as described above. The DNA was air-dried and re-suspended in water.
Dephosphorylation of vectors
Following restriction digestion, all vectors were treated with alkaline phosphatase (1 U/pmol 5’ ends) to eliminate 5’-end phosphate groups and prevent re-ligation of the plasmid. The enzyme, along with the corresponding buffer were added to the digest and incubated for a further hour at 37°C. The reaction was stopped by addition of 5x DNA sample buffer.

Agarose gel electrophoresis
DNA fragments were separated using TAE agarose gels for preparative and TBE agarose gels for analytical experiments. The desired amount of agarose was dissolved in TAE (National Diagnostics) or TBE buffers in the microwave and Ethidium bromide was added (40 μg/ml). Electrophoresis was carried out at 100 V for 60 min using two molecular weight standards (100bp and 1kb). The results were visualized under UV illumination (365 nm) and documented in printed form.

<table>
<thead>
<tr>
<th>10x TBE</th>
<th>Tris-Borat 890 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA 20 mM</td>
<td>Borat 890 mM</td>
</tr>
</tbody>
</table>

Gel extraction of DNA
The DNA of interest was isolated from the agarose gel using the Nucleospin Extract kit according to the manufacturers instructions. Following gel excision, the DNA-containing agarose block was dissolved at 56°C in 300 μl NT1 buffer/100 mg agarose. The DNA was extracted from this solution using a DNA binding column, subsequently washed twice with NT3 buffer and eluted with pre-warmed (70°C) NE buffer.
Polymerase chain reaction (PCR)

DNA amplification was carried out in 0.2 ml PCR tubes using a thermal cycler 2400 (Applied Biosystems). The reaction conditions varied according to specific experimental requirements.

**KOD Hot Start PCR** - The PCR was performed in a total volume of 50 µl using 1 U of the KOD-HotStart DNA polymerase in combination with 5 µl 10x KOD HotStart buffer, 40 ng template DNA, 50 pmol/µl of each primer (P2207 and P2208), 200 µM dNTP mix, 1 mM MgSO₄. After an initial denaturation of 30 sec at 94°C the PCR was performed with 15 cycles of 30 sec at 94°C, 30 sec annealing time at 50°C and 1.5 min elongation step at 72°C; following 7 min of additional elongation time at 72°C, the reaction was held at 4°C.

**Oligonucleotide-directed mutagenesis** - For the mutagenesis FLAG-hTRPM2 was cloned into Bluescript KS+(II) vector. Complementary primers containing the desired mutation (P2201 and P2202) were used to introduce the base pair change and amplify the entire plasmid by PCR using Pfu Turbo DNA polymerase (2.5 U). In a 0.2 ml PCR tube, 20 ng template DNA was combined with 5 µL 10x reaction buffer, 250 µM dNTP mix and 125 ng of each primer in a total volume of 50 µl. After an initial denaturation of 5 min at 95°C, the PCR was performed with 12 cycles of 30 sec at 95°C, 1 min annealing period at 50°C and an elongation step of 8 min at 68°C extended by 10 s in every cycle. The PCR ended with an additional 7 min synthesis step at 72°C and 10 min at 40°C, at which point the reaction was held at 4°C until removed from the cycler. The reaction products were analyzed by agarose gel electrophoresis and the residual amplification product was digested with DpnI. 20 % of the digest was transformed into DH5α.
**Sequencing** - Sequencing reactions were performed in a total volume of 20 µl made up of 4 µl BigDye Terminator kit v1.1, 135-200 ng plasmid DNA and 3.2 pmol sequencing primers. After an initial denaturation at 94°C for 30 sec the PCR was performed with 25 cycles of 30 sec at 94°C, 15 sec annealing time at 50°C and 4 min elongation period at 60°C. Final elongation was 7 min at 72°C upon which time the reaction was held at 4°C.

The PCR products were precipitated for 10 min on ice in the presence of 300 mM NaAc (pH 4.6) and 50 µl EtOH (100 %), followed by centrifugation at 15200xg for 15 min (4°C). The pelleted DNA was washed with 250 µl EtOH 75 % followed by centrifugation (see above). Finally, the pellet was air-dried and the samples were sent for analysis in the departmental sequencing facility (ABI310 sequencer). Sequencing data were examined using the DNAstar software (Lasergene).

**Ligation**

Ligation of DNA fragments into dephosphorylated vectors was carried out by combining the T4 DNA Ligase (1 U), 5x ligation buffer, 20 ng of vector and DNA insert (3 molar excess) in a total volume of 20 µl. The reaction mix was incubated in a water bath at 14°C for 1-2 hours.

5x Ligation Buffer
- MgCl₂ 50 mM
- Dithiothreitol 50 mM
- ATP 5 mM
- Tris-HCl 100 mM
- pH 7.5

**Transformation into DH5α E. coli**

Recombinant DNA was transformed into competent DH5α E. coli bacteria kept at -80°C. Aliquots of competent bacteria (100 µl) were thawed and mixed with 10 µl of the ligation reaction or DNA to be transformed. The mixture was
incubated first on ice for 15 min and then in a heat block at 37°C for 5 min. After this, 200 µl of pre-warmed LB medium were added and the mix was returned to the heat block for a further 15 min. The transformation mix was evenly spread onto pre-warmed LB/Ampicillin agar plates and incubated overnight at 37°C, 5 % CO₂. To perform a blue-white selection 40 µl IPTG (100 mM) and 10 µl X-Gal (30 mg/ml) were distributed on the LB-Ampicillin plates before plating the bacteria.

<table>
<thead>
<tr>
<th>Miller’s Luria Broth bacterial (LB) medium</th>
<th>dH₂O 1L</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB/Ampicillin Medium</td>
<td>Luria Broth 25 g</td>
</tr>
<tr>
<td>denomination</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Plasmid DNA purification - small scale**

A single colony was inoculated in 4.5 ml LB/Ampicillin medium. Cultures were allowed to grow overnight in a shaker incubator at 37°C, 5 % CO₂, 220 rpm. Plasmid DNA was isolated using the NucleoSpin Plasmid kit following the manufacturer’s instructions. Cells were pelleted by centrifugation at 3200xg for 5 min. The supernatant was removed and the pellet re-suspended in 250 µl buffer A1. The suspension was transferred to a 1.5 ml tube and the cells were lysed using 250 µl buffer A2. Subsequently, 300 µl of the neutralizing buffer A3 was added and the cell debris was collected by centrifugation at 16000xg for 5 min. The supernatant was loaded into the plasmid DNA-binding NucleoSpin column and centrifuged for 1 min at 10000xg. The DNA was washed with 600 µl buffer A4, centrifuged at 10000xg and dried with full speed centrifugation at 16000xg. The DNA was eluted by addition of 50 µl prewarmed buffer AE (70°C), incubation at RT for 1 min and centrifugation at 16000xg for 1 minute.
Plasmid DNA purification - medium scale

A single colony was inoculated in 40 ml LB/Ampicillin-medium and grown overnight in a shaker incubator at 37°C, 5 % CO₂, 220 rpm. Plasmid DNA was isolated using the NucleoBond PC 100 kit according to the manufacturer’s instructions. Bacteria were pelleted by centrifugation at 3200xg for 10 min. The pellet was re-suspended in 4 ml buffer S1 and lysed by addition of 4 ml buffer S2. Following this, 4 ml buffer A3 was added and cell debris was pelleted by centrifugation at 3200xg for 5 min. The supernatant was filtered and the clarified lysate was loaded onto a NucleoSpin AX 100 column, equilibrated with 2.5 ml N2 buffer. The column was washed twice with 5 ml buffer N3. Plasmid DNA was eluted using 5 ml buffer N5. DNA was precipitated by addition of 3.5 ml isopropanol and centrifugation for 30 min at 12000xg (4°C). The pellet was washed with 2 ml 75 % EtOH followed by centrifugation at 12000xg for 10 min (4°C). The DNA was air-dried and re-suspended in distilled H₂O at a final concentration of 1 µg/ml. DNA purity and concentration were determined using a NanoDrop Spectrophotometer.

2.2.4 Cell transfection

Transient transfection of HEK 293

HEK cells were plated onto 35 mm dishes and allowed to grow up to 90-95 % confluency. Cells were fed with fresh HEK complete medium 3-5 hours prior to transfection, after which time the medium was replaced with 1 ml OPTI-MEM. The DNA (total 1 µg) and Lipofectamine 2000 were diluted in parallel at a ratio of 1:2.5 in 100 µl OPTI-MEM and incubated for 10 min at RT. Subsequently the solutions were mixed together, incubated for a further 30 min at RT and added
to the cells. These were returned to the incubator (37\(^0\)C, 5 % CO\(_2\)) for 5 hours when the transfection medium was replaced with 2 ml HEK complete medium. EGFP-pcDNA3 coding for EGFP was co-transfected at a ratio of 1:3 with the DNA of interest in order to distinguish transfected from non-transfected cells. The DNA of interest were FLAG-hTRPM2-pcDNA3 and FLAG-hTRPM2-E960Q-pcDNA3, transfected separate or simultaneously in a 1:1 ratio. In the case of single transfections the plasmids were mixed in a 1:1 ratio with the empty pcDNA3 vector, in order to ensure that expression of TRPM2 and TRPM2-E960Q was comparable to that in co-transfection experiments. Cells were used for experiments 24 hours post-transfection.

**Transient transfection of hippocampal neurons**

Hippocampal neurons were used for transfection from DIV10-17. The DNA (total 1 µg) and Lipofectamine 2000 were diluted in parallel at a ratio of 1:1 in 25 µl OPTI-MEM and incubated for 5 min at RT. The solutions were subsequently mixed and incubated for an additional 20 min at RT. Half of the neuronal growth medium in each well (usually ~ 220 µl) was collected in a fresh 10 ml screw cap tube and the transfection mix was added to the cells. These were returned to the incubator for 2 hours (37\(^0\)C, 5 % CO\(_2\)) following which time the medium was carefully removed and replaced with 500 µl pre-warmed and enriched maintenance medium (220 µl collected from each well prior to transfection + 260 µl fresh). As described for HEK 293 cells, EGFP-pcDNA3 was co-transfected in all instances at a ratio of 1:3 with the DNA of interest. The latter was either FLAG-hTRPM2-pcDNA3, FLAG-hTRPM2-E960Q-pcDNA3 or pcDNA3 vector. Cells were used for experiments 24 hours post-transfection, having previously verified and documented the transfection efficiency using an
inverted Leica microscope equipped with a EGFP filter set, 40x lens, C4742-95 Hamamatsu camera and OpenLab software.

2.2.5 Electrophysiology

Recordings in primary cultured neurons

Whole-cell recordings were obtained under visual control (Axiovert 200, Zeiss) from morphologically identified pyramidal neurons in heterogeneous primary hippocampal cultures using the method described elsewhere (Hamill et al., 1981). An EPC10 patch clamp amplifier and Pulse v8.8 software (HeKa Electronik, Germany) were used for voltage and current clamp recordings, as well as 3-5 MΩ borosilicate glass capillaries pulled using a DMZ-Universal horizontal puller or an L/M-3P-A vertical puller. Electrodes were filled with a potassium gluconate-based intracellular solution. The liquid junction potential error under these conditions is 15.9 mV and was not corrected for these recordings. Cells were bathed with various extracellular solutions using a gravity perfusion system at 2 ml/min. Given the high level of synaptic activity of these neurons, all recordings were obtained in the presence of 25 μM NBQX and 5 μM APV to block AMPA and NMDA receptor activity (unless otherwise specified). Bath temperature was maintained at 27°C (except for temperature dependence experiments) using an in-line heating device directly connected to the recording chamber inlet and an associated digital feedback mechanism including a temperature probe reporting values in the centre of the chamber. The microscope, equipped with a UV lamp and a longpass emission filter, allowed the identification of EGFP-expressing neurons when required. The experimental solutions used are detailed below (in mM) and called out in the text as appropriate:
Current clamp

Upon establishment of the whole-cell configuration, the physiological state of all cells was verified with a brief switch to current clamp conditions. After taking note of the membrane resting potential ($V_{rest}$; acceptable range -50 to -70 mV) a series of 1 s long current injections in 50 pA steps was delivered to the cell in order to verify spiking activity. In one set of experiments, the amplitude of the voltage sag due to the activation of the hyperpolarization-activated current $I_h$ was measured using progressively increasing hyperpolarizing current injections (10 pA steps). The amplitude of the voltage sag was measured as the voltage difference between the peak of the voltage response to -90 pA of current injection and the steady state voltage value just before the termination of the pulse. Traces were acquired every 10 s and filtered at 2.8 KHz.
Voltage clamp

Series resistance, input resistance and capacitance - In voltage clamp recordings, the cells’ potential is held at a desired value (holding potential, HP) and current flowing through the membrane is recorded directly. In the whole-cell configuration, any current will flow through an arrangement of electronic components (biological and non-biological) referred to as the equivalent RC circuit. The resistive properties of this circuit are determined by the combined actions of the membrane resistance ($R_{in}$) and the series resistance ($R_s$), whilst the lipid bilayer of the plasma membrane affords capacitative features ($C_m$) to the system, thereby imposing an exponential delay in the voltage changes. The $R_{in}$ (usually >100 MΩ) is due to the activity of ion channels at rest and represents the largest resistor opposing whole-cell currents; $R_s$ (usually 10-50 MΩ in a neuron) is due to the pipette and access resistances, both of which are directly linked to the physical dimensions of the pipette and membrane openings which allow an electrical connection between the cell and the patch clamp amplifier. Because $R_{in}$ and $R_s$ are connected in series, the voltage drop across $R_s$ determines that the command voltage will be different from the potential difference achieved at the cell membrane (voltage drop through $R_{in}$), in other words, $R_s$ limits the efficiency of the voltage clamp. This voltage error was not compensated for in any of the recordings presented in this study given the relative voltage-independent nature and slow kinetics of the currents under study. However, considering the added complexity of a pyramidal neuron’s RC circuit, given mainly by its extensive dendritic arborizations and associated space clamp issues (additional voltage drops related to cable properties of the dendrites), $R_s$ values were maintained between 15-25 MΩ in all experiments. Cells with $R_s$ changes larger than 20 % were excluded from analysis.
In order to measure $R_s$, $R_{in}$ and $C_m$ in the absence of active conductances, neurons were voltage-clamped at -50 mV and a 100 ms, 5 mV hyperpolarizing step was delivered from that HP. Traces were recorded every 4 s and filtered at 5 kHz. Figure 4 provides details of these calculations using an example response to the above stimulus.

**Figure 4.** Measurement of passive membrane properties of the cell. A) Whole-cell current response (top) to a 5 mV hyperpolarizing voltage step ($V_{com}$, bottom) illustrating the fast capacitive transients at the onset and offset of the stimulus. B) Expanded current trace and measurements corresponding to the calculation of $R_{in}$ and $R_s$ using Ohm's Law (Equations 1 and 2: $V_{com} = \text{amplitude of command voltage step, } I_1 = \text{amplitude of the capacitive transient, } I_2 = \text{amplitude of the steady state current}$) and membrane capacitance ($C_m$, equations 3 and 4: $\tau = \text{membrane time constant, } Q = \text{charge accumulated by the capacitor}$). Eq. 3 was used when the decay of the capacitative transient was adequately described by a single exponential function.

**Recordings of $I_{AHP}$ and $I_{ADP}$** - Cells were voltage-clamped at -60 ($I_{ADP}$) or -50 ($I_{AHP}$) mV and stimulated with 200/300 ms-long depolarizing steps to +20/+30 mV. A large unclamped current, generally indicative of a net inward flow of Ca$^{2+}$ ions (‘Ca$^{2+}$ spike’) through voltage gated Ca$^{2+}$ channels (VGCC), was recorded as a result (Fig. 5 A). The consequent increase in intracellular Ca$^{2+}$ activates Ca$^{2+}$-dependent conductances that are visualized as tail currents upon the termination of the stimulus. Voltage protocols were optimized in terms of amplitude, duration and frequency, according to the developmental stage of the
neurons so as to obtain maximal and stable $I_{ADP}$ or $I_{AHP}$ amplitudes at each DIV. The stimulating protocol was delivered every 30 s and current traces were filtered at 250 Hz. At the beginning of each protocol, a 100 ms-long, -5 mV step was delivered to the cell, allowing monitoring of $R_s$ changes throughout the experiment. The Na$^+$ channel blocker tetrodotoxin (TTX, 0.5 μM) and the voltage-gated K$^+$ channel blocker tetraethylammonium (TEA, 1 mM) were routinely used in order to maximize Ca$^{2+}$ influx. In the case of $I_{ADP}$, a mix of pharmacological suppressors (XE991, 5 μM; d-Tubocurarine, 50 μM) of currents underlying the mAHP were also added to the extracellular solution, whilst the recording electrode contained 50 μM of the cyclic AMP analogue 8-(4-chlorophenylthio)-cAMP (8CPTcAMP), used to inhibit the sI$AHP$. Figure 5 provides details of the analysis of such recordings.

To determine the approximate current voltage relationship for $I_{ADP}$, neurons were stimulated with the depolarizing voltage step and subsequently held at voltage values from -90 to -10 mV for 400 ms before returning to the HP (-60 mV). The protocol was applied in the presence and absence of extracellular Ca$^{2+}$ to enable off-line isolation of the Ca$^{2+}$-dependent current under study by subtracting residual voltage-dependent components of the signal (as recorded in zero Ca$^{2+}$ conditions) also present in these cells.

Temperature dependence of $I_{ADP}$ was assessed with 3°C temperature steps from 24 to 33°C. Where possible, the 10-degree temperature coefficient was determined for $I_{ADP}$ current amplitude using the following equation (Kimura and Meves, 1979):

$$Q_{10} = \left( \frac{I_2}{I_1} \right)^{10/(T_2-T_1)}$$

where $I_1$ and $I_2$ are the $I_{ADP}$ amplitudes at temperatures $T_1$ and $T_2$, respectively.
Figure 5. Recording of $I_{\text{AHP}}$ and $I_{\text{ADP}}$ and measurement of current properties. A) Schematic representation of the voltage clamp protocol used (bottom: -5mV pre-step for monitoring $R_e$ and large depolarizing step) and resulting membrane currents associated with the activation of voltage- and Ca$^{2+}$-dependent conductances in the presence of TTX 0.5 µM and TEA 1 mM. B) Representative $I_{\text{AHP}}$ and $I_{\text{ADP}}$ current traces. Measurements were performed using the following criteria: current amplitude, amp: mean current value at the positive ($I_{\text{AHP}}$) or negative ($I_{\text{ADP}}$) peak of the tail current trace (usually ~100 ms after the termination of the depolarizing pulse for $I_{\text{AHP}}$ and between 50-70 ms for $I_{\text{ADP}}$); charge transfer, Q: area under the curve measured from the peak to 1.5 s after the termination of the pulse. Duration, dur: time from peak to full decay of the current. All values reported in the thesis represent averaged data from at least 3 traces.

Recordings in HEK-TRPM2 and HEK 293 cells

HEK-TRPM2 and HEK 293 cells were split, plated onto sterilized coverslips and allowed to recover in the incubator for 4-5 hours before recordings. Using the equipment already described, whole-cell recordings were obtained from isolated cells with 1.2-2.8 MΩ pipettes filled with a KGlucose-based intracellular solution including or not the TRPM2 channel agonist adenosine diphosphoribose (ADPR). Bath temperature was maintained at 24°C and
perfusion speed was 3 ml/min. Intra and extracellular solutions used are detailed below:

<table>
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<th>IS-3</th>
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<tbody>
<tr>
<td>KGluc</td>
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<tr>
<td>KCl</td>
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</tr>
<tr>
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</tr>
<tr>
<td>HEPES</td>
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<tr>
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<table>
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</tr>
<tr>
<td>NMDG</td>
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<td>5</td>
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<tr>
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<tr>
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</tr>
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<td>10</td>
</tr>
<tr>
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</tr>
<tr>
<td>Osmolarity</td>
<td>305-10</td>
<td>305-10</td>
</tr>
</tbody>
</table>

**Voltage clamp**

**Series resistance and capacitance** - When ADPR was present in the pipette, currents developed almost instantly upon achieving the whole-cell configuration. Series resistance and capacitance were therefore assessed very soon after break-through using a 5 mV hyperpolarizing step from a holding potential of +10 mV lasting 100 ms. Traces were sampled every 2 s at 25 KHz and analysis performed offline using the capacitive transient at the onset of the response as indicated for cultured neurons. Average series resistance was below 8 MΩ.

**Current-voltage relationships** - Whole-cell currents were measured using 100 ms-long voltage ramps from -140 to +120 mV every 5 ms, sampled at 2.5 KHz. Cells were held at +5 to +15 mV in order to minimize current flow through the membrane during non-stimulation periods. When recording leak currents (such as those mediated by TRPM2 channels in the presence of ADPR) large voltage
errors arise due to the fact that $R_s$ and $R_in$ are both low and within the same range. Large pipettes were used to minimize the effects of access to the cell and the remainder voltage error was compensated for offline using the principle of the voltage divider applied to the whole-cell currents observed. The corrected voltage values were calculated using the following equation:

$$corrected\ voltage = V_{com} \cdot \frac{R_{tot} - R_{pip}}{R_{tot}}$$

where $V_{com}$ is the command voltage, $R_{pip}$ is the pipette resistance and $R_{tot}$ represents the total resistance of the system ($R_s + R_in$), which can be calculated from the command voltage and measured current values using Ohm's Law.

Additionally, a liquid junction potential error of 15.8 mV was corrected for the generation of accurate IV curves. Inward and outward current amplitudes were measured at -60 and +40 pA and reversal potential values were obtained directly from the curves. Upon inhibition or block of TRPM2 currents, residual currents were analyzed in order to select the best recordings. The maximum permissible size of this residual ‘leak’ current was set at 10 % of the total whole-cell current.

**Recordings in hippocampal slices**

Brain sections were allowed to rest for 1 hour in a surface chamber under oxygenated conditions before recordings. Neurons in the CA1 region of dorsal hippocampal slices were identified under the stage of an upright Axioskop microscope and whole-cell recordings were obtained using the blind method (Blanton et al., 1989) with an EPC9 patch clamp amplifier and Pulse v8.53 software (HeKa, Electronik, Germany). Long-shafted electrodes of 5 - 6 MΩ resistance were pulled from borosilicate glass using a PP830 vertical puller and
filled with a KGlucosinate-based intracellular solution (IS-1). Recording ACSF bubbled with 95 % O₂ and 5 % CO₂ was pumped through the chamber at ~ 2.5 ml/min. Bath temperature was regulated using a temperature control system with an in-line heater. Series and input resistance were calculated using the method described before. Series resistance (<20 MΩ) and liquid junction potential (13.6 mV) error were not compensated for.

<table>
<thead>
<tr>
<th>Recording ACSF</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>MgCl₂ 1.5 mM</td>
</tr>
<tr>
<td></td>
<td>KH₂PO₄ 1.25 mM</td>
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<tr>
<td></td>
<td>NaHCO₃ 25 mM</td>
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<tr>
<td></td>
<td>Glucose 16 mM</td>
</tr>
<tr>
<td></td>
<td>CaCl₂ 2 mM</td>
</tr>
<tr>
<td></td>
<td>pH 7.4-5 with CO₂</td>
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</table>

**Current Clamp**

Membrane resting potential was measured directly for all cells in current clamp mode and only neurons with -55 < V_rest < -65 were used for experiments. In all instances, current was injected to maintain the membrane potential at -60 mV. All current clamp recordings were performed at 30°C.

**Excitability** - Pyramidal neuron firing properties were studied using a family of progressively increasing current injections (10 x 50 pA steps), delivered at 10 s intervals and lasting 1 s. Traces were sampled at 2.5 KHz and analyzed for spike frequency (number of action potentials in the train; detection threshold: 0 mV) and early adaptation of spike frequency (reduction in number of spikes as a result of underlying hyperpolarization). The latter was quantified using three different analytical approaches, aimed at measuring overall changes in interspike interval (ISI) or spike number in the early phase of the train (first 275 ms). These methods were: coefficient of variation of the ISI, ratio of number of action potentials in the last/first 75 ms of the early train and ratio of last/first ISI. A
diagram of these calculations is provided together with the relevant figure in the Results section. Additionally, the depolarizing envelope and spike amplitude attenuation were quantified for the 1s long train by measuring respectively the ratio between the minimum voltage in the last/first ISI and the ratio between the last/first action potential amplitude (baseline to peak).

**Action potential properties** - Action potential properties were analyzed using a 5 ms-long current step of 5-10 pA in amplitude. Single action potentials elicited with the minimum current injection (500-800 pA) were used for analysis. Traces were acquired every 10 s and sampled at 5 KHz. Threshold voltage was defined as the inflexion point at 10 % of the peak amplitude of the second derivative during the rising phase of the action potential. Other action potential features measured were: amplitude (baseline to peak), half width (duration at half maximal amplitude), spike ADP amplitude (measured from baseline to positive peak of membrane potential oscillation following the single action potential) and spike ADP duration (from peak to full decay of spike ADP).

**Afterpotentials** - The membrane potential shifts associated with the activation of Ca\(^{2+}\)-dependent currents were visualized under current clamp conditions following a 400 ms-long burst of action potentials elicited with 400 pA current injections. Five traces were acquired at an interval of 20 s, sampled at 1.56 KHz and averaged to facilitate measurements. The mAHP amplitude was quantified using the first afterhyperpolarizing peak in the membrane voltage response following the burst of spikes. The integral of the curve described by the voltage response post-burst was also analyzed as a measure of overall afterpotential magnitude.
Voltage clamp
Voltage steps to +10/+20 mV and 100 ms in duration were delivered from a HP of -60 mV in order to elicit Ca$^{2+}$ influx through VGCC. $I_{ADP}$ was observed following the depolarizing step in the presence of TTX 0.5 µM, TEA 1 mM, XE991 5 µM and d-Tubocurarine 100 µM. Series resistance variations were monitored throughout the experiment using the -5 mV step described previously. $I_{ADP}$ current measurements were performed as indicated for cultured neurons except the peak amplitude was often observed 40-60 ms after the termination of the pulse.

Drugs
All toxins and drugs used are listed in section 2.1.3. With the exception of Flufenamate (FFA), 2-aminoethoxydiphenylborinate (2APB), 4αPhorbol, Phorbol 12,13 dibutyrate (PDBu) and N-(p-amylcinnamoyl) anthranillic acid (ACA), which were dissolved in dimethyl sulfoxide (DMSO), drugs were dissolved in distilled H$_2$O and stored as stock solutions at -20°C, -80°C or 4°C. DMSO did not display any appreciable effect on $I_{ADP}$ at the working concentration (Fig. 6).

Data Analysis and Statistics
Analysis of electrophysiological data was performed using Pulsefit v 8.5 (HeKa, Electronik, Germany), Igor Pro 4.02, 6.12 (WaveMetrics Inc., USA) and Microsoft Office Excel (Microsoft Co., USA). Statistics were processed in InStat 2.03 and GraphPad Prism 4.0 (GraphPad Software Inc., USA); results are presented as mean ± SEM.

Paired or unpaired two-tailed Student t-tests were used where appropriate for statistical comparisons. In all cases the data was tested for normality and equal
Figure 6. Effects of extracellular DMSO on I_{ADP} current amplitude. A) Example I_{ADP} traces illustrating the lack of effect of the solvent on general current features. B) Time course of the experiment in A. C) Summary box plot of the data (n=7).

variances; where Welch-corrected t-tests or Mann-Whitney non-parametric analyses have been used, this is indicated in the text. In the case of multiple measurement comparisons a one-way analysis of variance test was used (one-way ANOVA). For comparisons involving the effects of two factors (e.g. whole-cell time and presence/absence of intracellular drugs) on a particular experimental response (e.g. current amplitude), a two-way Analysis of Variance (two-way ANOVA) with corresponding post-tests was used, usually based upon an un-weighted means analysis. The overall level of significance was defined as p<0.05.
Chapter Three

Results
3.1 I\textsubscript{ADP} in primary cultured hippocampal neurons

3.1.1 Properties of hippocampal pyramidal neurons in primary culture

Cultured neurons are explanted and exposed to a radical enzymatic and mechanic treatment that might affect the structural and functional integrity of membrane receptors and channels; this, as much as any other aspect in the physiology of these cells, requires careful examination during experimental design. Initial experiments were aimed at testing the use of primary cultures of hippocampal neurons as an appropriate system to study I\textsubscript{ADP}.

Whole-cell patch clamp recordings were obtained from 363 pyramidal-looking neurons from DIV 4 – DIV 30 (Fig. 7 A-C; 2.2.5: ES-1 and IS-1). One challenge encountered working with these heterogeneous cultures was the correct identification of the neuronal cell type to record from. Although cells will retain their approximate size in culture, one cannot assume that morphological types will be identical to those \textit{in vivo}. Nevertheless, in this case, it appeared relatively straightforward, at all ages of the culture studied, to distinguish pyramidal cells (clearly triangular-shaped somata with conspicuous and extensive neurites; Fig. 7 A-C) from interneurons or glial cells, the latter two presenting typical growth patterns in culture with few and short projections, oval or rounded cell bodies and flattened appearance (Banker and Goslin, 2002). The general changes in neuronal shape and size observed throughout the different DIVs, as well as the underlying physiological maturation of these cells are matched closely by the steady rise in cell capacitance, up to 5 mV hyperpolarizing shift of the resting membrane potential and dramatic drop in the input resistance observed as the time in culture increased (Fig. 7 D-F). Despite the fact that these passive membrane features presented some overall variability even
within the same DIV, they could be reliably used in the systematic selection of neurons for electrophysiological recordings, since it was not infrequent to find

Figure 7. Development of primary cultured hippocampal neurons from P0 rats. A, B, C) Characteristic morphology of cultured pyramidal neurons (indicated by arrows) at 3 distinct developmental stages (from left to right: DIV 4, 9 and 13). Pictures were taken using Leica Modulation Contrast and a 40X lens. Scale bar: 25 μm. D, E, F) Changes in the passive electrophysiological properties of visually identified pyramidal neurons from DIV 6 to 19 (n=7-50, 7-47 and 6-31, respectively; p<0.0001 in all cases, one way ANOVA). G, H, I) Representative current clamp traces illustrating the maturation of the firing behaviour of cultured neurons (from left to right: DIV 6, 9 and 14). Action potentials were generated in response to a 100 pA depolarizing current step in the presence of 25 μM APV and 5 μM NBQX.

different stages of development within the same coverslip. The highest patch clamp success rate was obtained between DIVs 10 and 19, which was
designated as the optimal period for stable recordings. Across these ages, average membrane capacitance was 165.5 ± 4.4 pF, n=147, and the resting potential and input resistance stabilized at -60.5 ± 0.3 (n=257) mV and 464.9 ± 19.7 MΩ (n=152), respectively.

Active membrane properties were equally variable and this reflected most evidently in the firing behaviour of these neurons, which consisted of single, burst, regular or irregular spiking, or a combination of these. In general, it was possible to differentiate immature and mature neurons according to their spiking (Fig. 7 G-I). Pyramidal neurons at early stages of development readily fired trains of action potentials in response to remarkably low current injections. That is a series of mostly overshooting spikes of variable duration usually followed by more immature action potentials and a period of membrane oscillations (Fig. 7 G). This behaviour was short-lived and older neurons in culture always fired overshooting actions potentials (Fig. 7 H, I) lasting under 2 ms. More specific features such as spike adaptation were also present from early on, but became more apparent at later stages. It is to be noted that these cultures exhibit a high degree of synaptic connectivity and this brings about spontaneous firing from around DIV 7. It was impossible to obtain any reliable and stable recordings thereon without the use of synaptic blockers (see 2.2.5).

3.1.2 $I_{\text{ADP}}$ unmasking in hippocampal cultures

Next I attempted to identify $I_{\text{ADP}}$ in primary cultures of hippocampal neurons and determine whether some of the features of $I_{\text{ADP}}$ initially described in hippocampal slices were retained by the primary culture preparation. This involved establishing the best conditions to elicit and stably record $I_{\text{ADP}}$ in this new model system.
Neurons were clamped at -60 mV in order to achieve a smaller driving force for K⁺ and potentially maximize the amplitude of I_{ADP}. Following depolarization of the membrane for 200-300 ms, an inward tail current was observed upon application of the blockers d-Tubocurarine (dTC, 50 µM) for I_{AHP} and XE991 (5 µM) for I_{M} (Fig. 8 A-C). Recordings were always performed under conditions of full Na⁺ channel block, partial block of voltage-gated K⁺ channels and complete inhibition of the sI_{AHP} (see 2.2.5).

Unmasking of I_{ADP} took place within a minute of application of the I_{AHP} and I_{M} blockers, whilst average time for run up and full stabilization of the current was 4 minutes (Fig. 8 D). Recordings were stable for 15-20 minutes, following which time a steady run down of the current was often observed, particularly if the
series resistance was kept below 20 MΩ. The current had kinetic features similar to those observed in acute slices. Though not always visible, $I_{ADP}$ peak in mature neurons was clearly distinguishable from the capacitative transient and occurred within 50-70 ms of termination of the depolarizing pulse ($I_{ADP}$ amplitude = 166.9 ± 4.4 pA, DIV 10-19).

Approximately 2 % (8/363) of neurons did not display an $I_{ADP}$ upon suppression of the outward $K^+$ currents mediating AHP. It was noted that these neurons had clearly lower input resistances (100-150 MΩ) and a very prominent, outward conductance (~ +40 pA), which might have partly or totally concealed $I_{ADP}$. These cells could represent a subpopulation of interneurons within the culture and were excluded from further analysis.

$I_{ADP}$ was observed throughout the developmental stages investigated (DIV 4-30); however, insufficient data has been gathered for DIVs below 6 and above 19, which represent either very immature cultures or late stages of development, the latter generally dominated by glia proliferation in vitro. Although quite variable, the average current density and duration remained relatively stable during the less vulnerable stages DIV 6-19 (Fig. 9), indicating that the current is expressed from early on in these neurons. An apparent increase in the duration was seen until DIV 10 followed by a relative stabilization in the more mature neurons (DIV10-19), where the mean $I_{ADP}$ density was 1.12 ± 0.03 pA/pF (n=147) and the current lasted 834.3 ± 27 ms (n=145). Current duration was measured because, in most cases, it was not possible to fit the decay of $I_{ADP}$ using an exponential function.
Figure 9. \( I_{\text{ADP}} \) current density (A) and duration (B) throughout culture development (n= 5-36 in both cases; respectively \( p=0.064 \) and 0.132, one way ANOVA). In (A) the boxplot was used to better illustrate the full data set and indicates: the median (small filled squares), 25th and 75th percentile of the data (upper and lower edges of empty boxes), and maximum and minimum values of the data (upper and lower limits of vertical lines).

### 3.1.3 \( \text{Ca}^{2+} \)-dependence of \( I_{\text{ADP}} \)

Substitution of extracellular \( \text{Ca}^{2+} \) (2 mM) with \( \text{Mg}^{2+} \) (5 mM) rapidly and reversibly abolished \( \text{Ca}^{2+} \) currents and \( I_{\text{ADP}} \) in all cells tested (Fig. 10; 2.2.5: ES-2). In many cases, a 5-20 pA \( \text{Ca}^{2+} \)-independent outward current became visible under these conditions. Removal of \( \text{Ca}^{2+} \) reduced \( I_{\text{ADP}} \) amplitude by 96.4 ± 2.3 % (n=37) within one minute of exposure to the high \( \text{Mg}^{2+} \) solution, which was kept to a minimum. The current recovered almost completely in all cases (98.1 ± 2.4 %, n=29).

This result indicated that the inward current observed was dependent on extracellular \( \text{Ca}^{2+} \). The next question was whether \( I_{\text{ADP}} \) was the result of the inward flow of \( \text{Ca}^{2+} \) through VGCC still open at the termination of the stimulus. To test this contention, the fast \( \text{Ca}^{2+} \) chelator BAPTA was included in the intracellular solution at a concentration of 10 mM (2.2.5: IS-2). This resulted in the complete inhibition of \( I_{\text{ADP}} \) within 3 minutes of establishing the whole-cell configuration in 7 out of 7 cells (Fig. 11 A, B). In contrast, \( \text{Ca}^{2+} \) spikes appeared slightly enhanced in the presence of BAPTA (Fig. 11 C).
Figure 10. Removal of extracellular Ca\(^{2+}\) readily suppresses I\(_{\text{ADP}}\). A-C) Representative current traces obtained when cells were superfused with an extracellular solution containing 2 mM Ca\(^{2+}\) (control, A), 0 Ca\(^{2+}\)/5 mM Mg\(^{2+}\) (B) and upon return to the initial conditions (recovery, C). Each I\(_{\text{ADP}}\) trace is accompanied by the corresponding Ca\(^{2+}\) spike to illustrate the concomitant changes in the unclamped Ca\(^{2+}\) currents, which precede I\(_{\text{ADP}}\) under the stimulating conditions used. D) Time course of the effect of removal of extracellular Ca\(^{2+}\) on I\(_{\text{ADP}}\) peak amplitude. E) Pooled data (p<0.001, paired t test).

Figure 11. Effects of intracellular BAPTA on I\(_{\text{ADP}}\) amplitude. A) Time course of the decrease in relative I\(_{\text{ADP}}\) peak amplitude as BAPTA diffuses into the cell upon establishment of the whole-cell configuration. B) Example I\(_{\text{ADP}}\) traces after 0 (i), 1 (ii) and 2.5 (iii) minutes of breaking into the whole-cell configuration. C) Ca\(^{2+}\) spikes preceding the I\(_{\text{ADP}}\) current traces in B (equal time scale) showing an increase in voltage-gated Ca\(^{2+}\) currents upon intracellular BAPTA diffusion.
In a separate set of experiments designed to establish the optimal protocol to obtain a maximal ADP current, it was evident that the amplitude of the current increased upon application of larger or more prolonged depolarizing steps (Fig. 12 A,C and B,D respectively). Equally, when Ca$^{2+}$ influx decreased with voltage steps beyond +70 mV or when longer-lasting (400-500 ms) depolarizations were applied, $I_{\text{ADP}}$ amplitude also declined.

Figure 12. $I_{\text{ADP}}$ amplitude changes as a function of pulse amplitude and duration. A, B) Representative calcium spikes (top) and $I_{\text{ADP}}$ traces (bottom) in response to depolarizing voltage steps of increasing amplitude (A) and duration (B, step to +20 mV) from a holding potential of -60 mV. C, D) Summary data, n= 3-14 and 5-14 respectively (p<0.001, one way ANOVA).

Because these experiments were performed at early as well as late stages of development, the data is presented normalized by cell capacitance. It can be concluded that gating of $I_{\text{ADP}}$ depends on the raise in intracellular Ca$^{2+}$ that
follows VGCC activity but is not a tail current through these channels.

### 3.1.4 Ionic nature of $I_{ADP}$

One important aspect in the correct identification of $I_{ADP}$ was to assess the nature of the ionic contributions to this current. Previous data pointed in the direction of $I_{ADP}$ being a mixed cationic conductance, which depends crucially on extracellular Na$^{+}$. This was tested by replacing all Na$^{+}$ ions in the extracellular solution with the larger and generally non-permeant ion NMDG$^{+}$ (2.2.5: ES-3 and ES-4), the culture preparation being more amenable to manipulations of this sort than brain slices.

The Na$^{+}$ free solution induced a fast decrease in both $I_{ADP}$ amplitude and charge transfer (98.6 ± 1.7% and 155.2 ± 19.5% respectively, p<0.0001 paired t test) and gave rise to an outward Ca$^{2+}$-dependent current of longer duration (Fig. 13 A, C). There were no associated input resistance fluctuations. In counterpoint to this result, perfusion of a high extracellular K$^{+}$ solution (7 mM, twice the regular amount; 2.2.5: ES-5) enhanced both $I_{ADP}$ amplitude and charge (19.2 ± 4.6 %, p=0.003 and 62.6 ± 21.1 %, p=0.01 respectively, paired t test). This larger $I_{ADP}$ was also fully abolished by removal of extracellular Ca$^{2+}$ (Fig. 13 B, D; 2.2.5: ES-6), although in this case, an outward long-lasting current persisted. Membrane input resistance dropped from 309 ± 24 MΩ to 194.2 ± 18.2 MΩ (p<0.0001, paired t test) upon exposure to high K$^{+}$. Returning to the regular extracellular solution fully reversed the effects in both ionic manipulations.

In order to complement these results, the reversal potential for $I_{ADP}$ under normal ionic conditions was determined, as well as any potential deviations in the absence of Na$^{+}$ or with a reduced K$^{+}$ gradient, as described above. $I_{ADP}$ was
recorded as usual, except the membrane potential was stepped to different values (from -90 to -10 mV) upon termination of the depolarizing pulse. This protocol was repeated for both zero Na$^+$ and high K$^+$ manipulations in the presence and absence of Ca$^{2+}$ in order to isolate the Ca$^{2+}$-dependent components from the voltage-gated currents also present in these neurons.

![Figure 13](image.png)

Figure 13. Na$^+$ and K$^+$ ions contribute to I$_{ADP}$. A) Example traces illustrating the characteristics of I$_{ADP}$ when extracellular Na$^+$ is completely replaced by NMDG$^+$ (green). The identity of the outward current observed under these conditions was verified by the additional removal of Ca$^{2+}$ (light grey); recovery (dark grey). B) Doubling of extracellular K$^+$ concentration enhances I$_{ADP}$ (brown) which is Ca$^{2+}$ dependent (light grey); recovery (dark grey). C, D) Summary diagram for the above experiments.
The $I_{ADP}$ current-voltage relationship in control solution was essentially linear. Thus the reversal potential could be easily predicted by extrapolation (Fig. 14, black). The current normally reversed around +18 mV. This value is shifted to -63 mV in the NMDG$^+$ solution and to +68 mV in the presence of high extracellular K$^+$ (Fig. 14, green and brown). The latter condition seemed also to affect the linearity of the IV curve at potentials positive to -40 mV. These points were excluded from the reversal potential analysis, since K$^+$ was previously shown to substantially affect membrane resistance. Instead, a more precise mapping of the IV was sought for the more hyperpolarized range of voltages, where the relationship was approximately linear. Overall, these results indicate that $I_{ADP}$ is a mixed cationic current to which Na$^+$ and K$^+$ ions make an important contribution as charge carriers.

### 3.1.5 $I_{ADP}$ is enhanced by moderate heat

Finally, the temperature sensitivity of $I_{ADP}$ in cultured neurons, which had previously been characterized in acute slices, was investigated. This property
constitutes an important link between \( I_{ADP} \) and potential candidates in the TRP superfamily of ion channels. Bath temperature was varied in steps of about 3\(^\circ\)C between room temperature (24\(^\circ\)C) and 33\(^\circ\)C. \( I_{ADP} \) amplitude consistently increased with temperature elevations (Fig. 15 A, B and D) and a concomitant decrease of the current duration was also observed (Fig. 15 C). The largest changes occurred between 27-30\(^\circ\)C: 170.1 ± 18.3 % increase in amplitude and 21.4 ± 2.1 % decrease in duration. Interestingly, the associated \( Ca^{2+} \) spike remained unchanged or was slightly reduced with higher temperatures (Fig. 15 A, top).

Figure 15. Temperature dependence of \( I_{ADP} \). A) Representative \( I_{ADP} \) traces and corresponding \( Ca^{2+} \) spikes showing the increase in peak amplitude and reduced duration of the current observed in response to 3-degree temperature steps: from 27\(^\circ\)C (black) to 30\(^\circ\)C (orange) to 33\(^\circ\)C (red). B) Time course of the increase in current amplitude for the cell in A. C) Mean changes in \( I_{ADP} \) duration with increasing temperatures (\( p=0.173 \), one way ANOVA). Only those cells where amplitude could be estimated from a visible peak, and not by extrapolation were considered. D) Summary of the increase in current amplitude (\( p<0.001 \), one way ANOVA).
The $Q_{10}$ coefficient was used as a measure of temperature sensitivity for both changes in amplitude and duration, but this was limited by the fact that recordings did not frequently withstand more than two consecutive temperature steps ($Q_{10} 24-30 = 3.4; Q_{10} 27-33 = 2.3; n=4$ in both cases).
3.2 Pharmacological profile of $I_{\text{ADP}}$

3.2.1 $I_{\text{ADP}}$ modulation by generic TRP channel drugs

In line with the general aim of identifying potential channels underlying $I_{\text{ADP}}$, I next undertook a candidate ‘refinement’ strategy centred on the pharmacological characteristics of the current in primary culture. The ultimate aim would be to interfere with the functional expression of potential candidate channels by means of specific pharmacological tools and genetic manipulation in the chosen model system.

A brief look into the potential contribution of the hyperpolarization-activated current $I_h$ revealed that, although mixed cationic in nature and present in hippocampal neurons (Vasilyev and Barish, 2002; Noam et al., 2010), HCN channels are unlikely to underlie $I_{\text{ADP}}$, since complete block by the specific blocker ZD7288, applied intracellularly to avoid unwanted effects on VGCC (Harris and Constanti, 1995; Sánchez-Alonso et al., 2008), resulted in the abrogation of the $I_h$-mediated voltage sag in cultured neurons without any appreciable disturbance of the Ca$^{2+}$-activated current of interest (Fig. 16). The general approach of the refinement strategy was therefore based on the premise that TRP channels are currently the most suitable candidates to underlie $I_{\text{ADP}}$.

As a starting point, the 17 hippocampal TRP family members were selected as the most likely mediators of $I_{\text{ADP}}$ on the basis of their expression profile (see Fig. 3, Introduction). Given the limited specificity of TRP drugs to date, it was determined to begin the study with compounds that would single out groups rather than individual TRP channels, or at the very least give a more direct indication that this is a suitable family of potential candidates. Thus, generic
TRP channel drugs with overlapping but sometimes opposing effects on different subfamilies of TRPs were selected (Fig. 3, highlighted text). All bath-applied drugs were perfused for the minimum period required to reach a steady state condition in the monitored parameters.

Figure 16. Effect of intracellular ZD 7288 on I_{ADP}. A) Current clamp traces illustrating the characteristics of the voltage response to a -90 pA step of 1 s in duration at the start of the experiment and 10 minutes after achieving the whole-cell configuration. B) Corresponding I_{ADP} recordings from the cell in A showing no qualitative alterations in the current upon diffusion of the I_{h} inhibitor. C, D) Summary diagrams for the changes in voltage sag amplitude and I_{ADP} current density during the first 10 minutes of recording (n=5 in all cases). Statistical analyses used were Mann-Whitney and unpaired t tests, respectively.

Figure 17 illustrates with example time courses and traces the effects observed with these compounds, whilst Figure 18 summarizes the relative changes in current amplitude observed. The non-steroidal anti-inflammatory drug flufenamate (or flufenamic acid, FFA) is widely recognized as a wide spectrum Ca^{2+}-activated non-selective cation channel blocker. At a concentration of
20 µM it produced a 52.1 ± 14.3 % inhibition of $I_{ADP}$ (n=4; Fig. 17 A and 18) upon perfusion for at least 6 minutes, which is a relatively long period of time when compared to the effects of other drugs in the dissociated culture (usually within 2 min). For this reason, although the decrease in current amplitude was observed in all 14 cells tested, in most cases it was not possible to assess the full extent of the action of the drug, as the recordings deteriorated. In addition, FFA produced a consistent inhibition of the unclamped Ca$^{2+}$ currents at 20 µM (n=8); halving the concentration of the drug did not seem to confer any specificity to its actions and reduced both $I_{ADP}$ (~60%; n=6) and the Ca$^{2+}$ currents to a similar extent. The effects of FFA were not reversible.

Figure 17. Effect of generic TRP channel drugs on $I_{ADP}$. A- F) Representative time courses of the changes in peak $I_{ADP}$ amplitude (left panels) during extracellular application of known TRP channel modulators. Shaded areas correspond to the time intervals of exposure to the drugs. Example $I_{ADP}$ current traces and Ca$^{2+}$ ‘spikes’ are placed to the right of each time plot and color coded as follows: control (black); drug (blue).
Figure 17B illustrates the reduction of both $I_{ADP}$ amplitude (40.7 ± 8.9 %, n=4; Fig. 18) and calcium spike observed with ruthenium red (RuR, 10 µM), best known as a blocker of TRPV channels. In this particular example, a Ca$^{2+}$-free solution was applied to show whether the non-blocked component was still Ca$^{2+}$-dependent (n=2). In relative terms, the full effect of RuR was reached rather fast, between 3-4 minutes. Like in the case of FFA, the effects of this drug were not reversible.

Lanthanum and gadolinium, which have been used extensively to identify TRP channel currents in various contexts, were tried next. In cultured neurons La$^{3+}$ and Gd$^{3+}$ (100 µM) respectively blocked 104.7 ± 5 % and 104.3 ± 9.5 % of the control $I_{ADP}$ (n=4 in both cases; Fig. 17 C, D and 18) almost irreversibly and within seconds of application. As exemplified in the figure, this was accompanied by a total abolition of the Ca$^{2+}$ current. At concentrations of 100 µM and above, these lanthanides normally act as TRP channel blockers. Instead, around 10 µM or below, they are capable of activating some TRPC channels (Ramsey et al., 2006). When applied on $I_{ADP}$ at this concentration, the result was similar to that obtained with 100 µM (104.6 ± 5.3 % inhibition with La$^{3+}$ and 108.4 ± 6.4 % with Gd$^{3+}$, n=3 in both cases), though this lower concentration proved slightly easier to wash out.

The compound 1-{$\beta$-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole (SKF96365) is known to target some channels of the TRP family, although, conveniently, it lacks effect on others (see Fig. 3, Introduction). At 50 µM, SKF96365 inhibited 89.3 ± 2.3 % of $I_{ADP}$ peak amplitude within 3.2 min (n=7) and, unlike the compounds described above it conspicuously enhanced the Ca$^{2+}$ spike area during the depolarizing pulse. However, the latter effect was
biphasic, in that the apparent increase in Ca\(^{2+}\) current was followed by a reduction in the peak of the unclamped spike. This prompted experiments with a lower concentration to verify that the overall effect of SKF96365 was a boosting of the Ca\(^{2+}\) spike and not a decrease, since this would have critical implications in the interpretation of the data. Thus, SKF96365 20 µM inhibited 72.4 ± 5.9 % of the control I\(_{ADP}\) (Fig. 17 E and 18; n=7), though the steady state was reached 1.6 min later. In this case only a boosting effect on the Ca\(^{2+}\) spike was observed. The actions of SKF96365 were easily, though not fully, reversible at both concentrations. Upon washout of the drug, the current recovered up to 53.1 ± 7.6 % (50 µM) and 87.1 ± 18.5 % (20 µM) of the initial amplitude.

The last generic compound tested, 2-aminoethoxydiphenylborinate (2APB), is a membrane permeable molecule of known inhibitory actions on TRPC, M and P. In contrast, it functions as an agonist in the case of TRPV channels. These possibilities were examined and the results were roughly similar to the effects of SKF96365 on I\(_{ADP}\). At both concentrations tested, 50 and 25 µM, application of 2APB led to a significant reduction of I\(_{ADP}\) (~53 %, n=6 and 34.3 ± 4.7 % respectively, n=5; Fig. 17 F and 18) together with an enhancement of the Ca\(^{2+}\) spike. Secondary reduction of the spike was not evident. However, the highest concentration was poorly tolerated by the neurons, and great series resistance changes prevented an accurate analysis of the results. As well as augmenting the Ca\(^{2+}\) spike, both concentrations of 2APB induced a much faster and short-lived inward current within the first 50 ms of stimulation (marked with an asterisk in Fig. 17 F). As shown in the representative trace, 2APB also affected the kinetics of I\(_{ADP}\), slowing down the decay by about 250 ms in 3 out of 5 cells; this was also observed in the remaining 2 cells tested at this concentration, but in
In this case the slower component (of less than 10 pA in amplitude) did not decay at all. In connection with this, it should be said that application of 2APB was always accompanied by a modest (-15.2 ± 3.5 pA) shift in the holding current, though no significant input resistance changes were observed (8.5 ± 8.5 % increase, p=0.8 paired t test). A recovery of 79.6 ± 5.4 % (n=4) was achieved upon washout of the drug.

Figure 18. Summary of the effects of generic TRP channel modulators on $I_{ADP}$ amplitude. Drug action is expressed as percentage current amplitude left upon reaching steady state conditions in the presence of the compound. Sample numbers and are specified within the graph. (p<0.01 in all cases, paired t test).

In summary, all of the TRP channel modulators tested on $I_{ADP}$ suppressed the current to a greater or a lesser degree when compared to the control values (Fig. 17). However, only in 2 out of 6 instances (SKF96365 and 2APB) have these effects been dissociated from a reduction in the influx of $Ca^{2+}$ into the cell.

3.2.2 Effects of TRPV channel agonists

The TRPV group of channel proteins is notorious for gathering the most versatile and broadly expressed members of the TRP family of ion channels; it is not surprising that they are also amongst the best characterized
pharmacologically. One particular set of compounds, the phorbols, are unique in their direct agonistic effects on several subtypes of TRPVs (see Fig. 3, Introduction). Considering the temperature dependence of \( I_{ADP} \) and in accordance with the results obtained with the broad spectrum TRP drugs, particularly SKF96365 and to some extent 2APB, I decided to test the effects of phorbols on the hippocampal \( I_{ADP} \).

The exogenous PKC activator Phorbol 12,13 dibutyrate (PDBu) was bath applied at a concentration of 5 µM. An increase in the current charge transfer (47.3 ± 8.3 %) and amplitude (16.3 ± 3.7 %) was evident within 1 min of exposure to PDBu (Fig. 19). As a result, \( I_{ADP} \) decay time was slowed by 354.8 ± 92.4 ms (\( I_{ADP} \) duration in PDBu = 1195 ± 191 ms, n=5, p=0.0185 paired t test).

Figure 19. Effect of the PKC activator PDBu on \( I_{ADP} \). A) Representative \( I_{ADP} \) current traces (bottom) and corresponding Ca\(^{2+}\) spikes (top) illustrating the actions of PDBu. B, C) Time course of the changes in amplitude and charge for the experiment in A. D) Pooled data expressed as percentage change with respect to control (n=5; paired t test).
No significant variations in input resistance were measured, although there was a tendency towards lower values (overall ~ 15% drop, p=0.15, paired t test). In 5 out of 5 cells, the boosting of $I_{\text{ADP}}$ was associated with an increased $\text{Ca}^{2+}$ influx, as judged by the change in the spike area (Fig. 19A, top). Particular care was taken that exposure to this phorbol ester was only as short as needed, given the possibility of cross-targetting of $\text{Ca}^{2+}$ channels in these neurons (Doerner et al., 1990). Despite this, $\text{Ca}^{2+}$ currents were reduced at the end of drug application and throughout the washout.

An inactive form of phorbol ester, 4αPhorbol, commonly used to identify non-PKC mediated effects of phorbols, produced an enhancement of $I_{\text{ADP}}$ in the same order as that observed with PDBu (amplitude: 11.4 ± 3.2 %; charge: 42.0 ± 5.4 %; Fig. 20).

Figure 20. Effect of 4αPhorbol on $I_{\text{ADP}}$. A) Representative $I_{\text{ADP}}$ current traces (bottom) and corresponding $\text{Ca}^{2+}$ spikes (top) illustrating the changes in $I_{\text{ADP}}$ upon application of 4αPhorbol. B, C) Time course of the changes in amplitude and charge for the experiment in A. D) Pooled data expressed as percentage change with respect to control (n=6; paired t test).
Likewise, a concomitant increase in the CA$^{2+}$ spike area was observed although no additional changes in these currents were recorded during removal or washout of the drug. $I_{ADP}$ decayed 200 ms slower than control ($n=5$, $p=0.18$, paired t test) and the recovery achieved was $73.1 \pm 4.1\%$ ($n=3$). Input resistance was not significantly affected. Moreover, it was determined (2 out of 2 cells) that the enhanced $I_{ADP}$ retained the characteristic sensitivity to extracellular Na$^+$, as the current was completely suppressed upon substitution of this ion with NMDG$^+$ also in the presence of 4αPhorbol (Fig. 20 A, grey).

### 3.2.3 Effects of TRPM channel modulators

Another important candidate group amongst TRP channels is the TRPM family, which includes both thermosensitive and highly Na$^+$ permeant channels. Although their pharmacological characteristics are less well defined than for the TRPVs (with the exception of TRPM8, which is not expressed in the hippocampus), there are a number of compounds whose actions set them apart. Initially, spermine and adenosine monophosphate (AMP) were selected because both have been used widely as blockers of TRPMs. However, since it is somewhat difficult to ascertain whether and to which extent intracellularly applied compounds have diffused into complex cells, it was decided to apply them at concentrations higher than those reported in the literature to be effective.

In the course of the experiments, there was no noticeable change in the development or shape of $I_{ADP}$ for either spermine (1 mM) or AMP (5 mM). Recordings were stable, including qualitative assessment of the CA$^{2+}$ spikes. However, after further analysis and normalization of the current amplitude or charge to either cell capacitance or as a fraction of the start value, it emerged
that spermine 1 mM inhibited the normal run up of $I_{\text{ADP}}$ within 2.5 min of diffusing into the cell. This effect is evident only for the area under $I_{\text{ADP}}$ (Fig. 21 A and B, control: n=3-9; spermine: n=3-7). No associated input resistance variations were noted (p=0.364, Mann Whitney test).

Figure 21. Effects of TRPM modulators on $I_{\text{ADP}}$. Average changes in $I_{\text{ADP}}$ amplitude (A and C) and charge transfer (B and D) as Spermine 1 mM or AMP 5 mM diffuse into the intracellular space: control (black), drug (blue). Results are presented normalized to the value measured at the outset of the recording and graphed as a function of time in the whole-cell configuration (two way ANOVA).

In the case of AMP, the result was even more surprising as the nucleotide did not produce the inhibitory effects expected. Instead, after 5 minutes in the whole-cell configuration it modestly enhanced both amplitude and charge transfer of $I_{\text{ADP}}$ (Fig. 21 C and D, control: n=3-9; AMP: n=8-16), without affecting the input resistance. These changes were statistically significant and did not involve additional alterations in the Ca$^{2+}$ spike.
In light of these puzzling results, I opted for more specific compounds known to target TRPM channels. Both N-(p-amylcinnamoyl) anthranillic acid (ACA) and ADP ribose (ADPR) have been extensively used in several heterologous and native expression systems to single out TRPM2-mediated events. In the presence of ACA (20 µM) $I_{\text{ADP}}$ peak amplitude slowly declined by 83.7 ± 3.0 % (Fig. 22). The average time of the effect was 6.2 min. Calcium currents were similarly suppressed, even upon wash out of the drug, allowing no recovery of $I_{\text{ADP}}$.

![Figure 22](image.png)

Figure 22. Effects of ACA (20 µM) on $I_{\text{ADP}}$ amplitude. A) Representative $I_{\text{ADP}}$ (bottom) and Ca$^{2+}$ spike (top) traces illustrating the decrease in both currents in the presence of the TRPM2 blocker. B) Time course of the changes in $I_{\text{ADP}}$ for the cell in A. C) Summary of the data: ACA n=6; wash n= 4; p<0.0001, paired t test.

ADPR is an endogenous nucleotide derived from the metabolism of nicotine amide dinucleotide (NAD). To date the only established target of this compound is the ion channel TRPM2, whose C-terminus region shares 50 % amino acid
sequence similarity with the ADPR hydrolytic enzyme NUDT9 (Perraud et al., 2001), thereby providing a high affinity binding site for this molecule.

When 300 µM ADPR was included in the pipette solution, a marked increase in current amplitude and duration was observed, transforming $I_{ADP}$ into a noisy, slowly deactivating conductance (Fig. 23 A, blue). Peak amplitude doubled and charge transfer grew up to 6 fold within the first 4 minutes of recording (Fig. 23 C, D). $Ca^{2+}$ spikes were often minimally enhanced, whilst a drastic reduction in the input resistance accompanied the development of this large current (Fig. 23 B). The effects of ADPR on $I_{ADP}$ are illustrated in Figure 23.
A, B). Out of 51 cells tested, only 2 (4%) did not display changes in the ADP current in the presence of ADPR. The actions of this drug were most dramatic, but also quite variable, thus maintaining the series resistance below 15 MΩ was critical to ensure the appropriate diffusion of the agonist into the cells. The effects of ADPR became central to the subsequent expansion of this project.

Overall, I_{ADP} responded to generic TRP channel modulators although, with the exception of SKF96365 and 2APB, drug effects were biased by the parallel suppression of voltage-activated Ca^{2+} currents. In analogy, the changes in Ca^{2+} spike associated with the presence of TRPV agonists make their effects on I_{ADP} amplitude and charge transfer a non-conclusive result. On the other hand, the combined action of the TRPM channel drugs Spermine and ADPR clearly establish the occurrence of a significant TRPM2-mediated current kinetically similar to I_{ADP}. 
3.3 Validation of TRPM2 as a candidate channel to underlie $I_{\text{ADP}}$

In the absence of selective blockers for TRPM2, it was essential to establish whether the large current evoked by ADPR was actually a potentiated form of $I_{\text{ADP}}$ and not due to the activation of a separate conductance with similar kinetics. Subsequent experiments were directed towards testing TRPM2 as a potential candidate for the molecular identity of this current in hippocampal neurons. For this, many of the experiments described in sections 3.1 and 3.2 were repeated, this time in the presence of intracellular ADPR 300 µM.

3.3.1 The ADPR mediated current is Ca$^{2+}$ and Na$^{+}$ dependent

Perfusion of a Ca$^{2+}$-free extracellular solution rapidly abolished the inward aftercurrent (amplitude: 97.1 ± 4.5 %, charge: 108.8 ± 7.2 %) and the accompanying Ca$^{2+}$ spike (Fig. 24 A). An outward current under 20 pA in amplitude and often long lasting was uncovered under these conditions, which, interestingly, was a lot less noisy that the inward ADPR mediated conductance. The full effect of Ca$^{2+}$ removal was reached within a similar time frame as previously shown for $I_{\text{ADP}}$ alone (i.e. 1.3 min) and was almost completely reversed (>95 % for amplitude and charge), although both Ca$^{2+}$ currents and $I_{\text{ADP}}$ were already declining by this point (Fig. 24 A-C). Moreover, when BAPTA 10 mM was added to the pipette solution, a full inhibition of the enhanced $I_{\text{ADP}}$ was observed (90 ± 3 % of initial amplitude, Fig. 24 D, E) although this was significantly delayed with respect to the same experiment in the absence of ADPR (5 minutes whole-cell instead of 3, p=0.006, Mann Whitney test). At this point, careful examination of the traces revealed the presence of large single channel-like events at -60 mV (Fig. 24 F, pink) crucially indicating that ADPR alone was not able to generate a macroscopic current of the dimensions
described in section 3.2.3 in the absence of raised intracellular Ca²⁺. In correspondence with the lack of effect of ADPR under these conditions, no significant changes in input resistance were observed in comparison with previous control recordings in the presence of BAPTA alone (R_{in BAPTA/ADPR} = 289

Figure 24. Extra and intracellular Ca²⁺ dependence of the ADPR mediated current. A) Representative I_{ADP} and Ca²⁺ spike traces showing the effects of replacing extracellular Ca²⁺ with Mg²⁺ on the current properties. B) Time course of the changes in I_{ADP} charge transfer; blue denotes presence of ADPR throughout the recording. C) Summary of the Ca²⁺ free effects. D) Average changes in peak current amplitude as a function of whole-cell time as BAPTA diffuses into the cell. Only those neurons where ADPR effect was observed are included in this analysis. (n=4). E) Example traces illustrating the effects of BAPTA (pink) on the augmented I_{ADP} and the Ca²⁺ spike (black). The area delimited by the dashed box is magnified in F.
± 27 MΩ, n=4 vs \(R_{in}^{\text{BAPTA}} = 358.4 \pm 38 \text{ MΩ, n=7; p=0.24 unpaired t test}\). The changes induced by BAPTA in \(I_{\text{ADP}}\) were accompanied by an increase in the \(\text{Ca}^{2+}\) spike area. Furthermore, as shown in Figure 25, exposure to a \(\text{Na}^+\) free (NMDG⁺-based; 2.2.5: ES-3) extracellular medium suppressed the inward component of the ADPR enhanced current, as observed previously for \(I_{\text{ADP}}\) alone. A total reduction in amplitude (103.2 ± 0.7 %) and charge transfer (111.5 ± 1.3 %) was observed.

![Figure 25](image)

Figure 25. \(\text{Na}^+\) dependence of the ADPR mediated current. A) Representative \(I_{\text{ADP}}\) traces in sequential order according to the different ionic conditions tried. B) Time course of the changes in \(I_{\text{ADP}}\) charge transfer for the cell in A. C) Summary of the effect of extracellular NMDG⁺ in 4 cells.

The remaining outward conductance (green) was similarly shown to be \(\text{Ca}^{2+}\) dependent (light grey; 2.2.5: ES-4), whilst restitution of the normal extracellular
ionic conditions allowed up to 60 % recovery of the initial size of the ADPR boosted $I_{ADP}$ (dark grey).

In summary, the ablation of the ADPR-induced current under conditions of zero extracellular and intracellular free $Ca^{2+}$ or zero extracellular $Na^+$ demonstrates that this current and $I_{ADP}$ share common features regarding activation mechanism and principal charge carrier.

3.3.2 HEK TRPM2 cells as a useful tool in the study of potential links between $I_{ADP}$ and TRPM2

As part of the efforts to draw a parallel between the neuronal ADPR-induced current, TRPM2 and $I_{ADP}$, I encountered an important limiting factor: many of the properties described for $I_{ADP}$ (such as temperature dependence and some pharmacological effects) had been poorly, if at all, studied for TRPM2, and vice versa. For this reason, I turned to a HEK cell line that expresses a FLAG-tagged version of TRPM2 when tetracycline is added to the growth medium (henceforth referred to as HEK-TRPM2 cells, see 2.2.1), which enabled the simultaneous analysis of some of these features in the isolated TRPM2 channel.

Using immunocytochemistry, it was initially verified that exposure to tetracycline indeed resulted in the overexpression of TRPM2 in these cells (see 2.2.2). The antibiotic was added to the culture medium at a concentration of 0.5 µg/mL and cells were returned to the incubator overnight. The immunoassay revealed Cy3 fluorescence in the cytoplasmic space of both induced and non-induced cells (Fig. 26 A, B). Although in the latter case this occurred at substantially reduced levels ($\leq 30$ % of cells in the dish vs. $>95$ % in induced cells), it was a surprising finding nonetheless.
Figure 26. Characteristics of TRPM2 channel expression in the tetracycline-inducible HEK cell line. A, B) Immunolabelling of FLAG epitope in HEK-TRPM2 cells with (+tet) and without (-tet) tetracycline pre-treatment. Scale bar: 20 µM C) Averaged time course of the whole-cell currents recorded from these cells in the presence of the TRPM2 agonist ADPR (-tet n=8; +tet n=3-20). D) Typical IV relationships of induced (red) and non-induced (black) cells. Dashed box is enlarged in E (black). E) Control recordings with ADPR from non-induced cells reveal the activation of a small voltage-gated outward current (black), which is less pronounced without the agonist (grey; n=3). F) Representative time course (inward currents measured at -60 mV; outward at +40 mV) showing the selective suppression of HEK-TRPM2 inward currents upon exposure to a Na⁺ and Ca²⁺ free extracellular, corresponding G) IV curves and H) summary data (0Na⁺/0Ca²⁺ n=30, recovery n=14; p<0.0001, paired t test). I) Time course of the block of HEK-TRPM2 inward and outward currents by ACA, corresponding J) IV curves and K) summary data (n=10; p<0.0001, paired t test).
On the other hand, electrophysiological recordings from these cells (2.2.5: ES-7, IS-3) presented a rather different scenario. In the presence of 30 µM ADPR only tetracycline-induced cells developed a whole-cell current with a characteristic TRPM2 profile (Fig. 26 C,D). The average recording lasted 400 s and the current was activated instantly upon breaking into the whole-cell configuration. This meant that cell capacitance (10.2 ± 0.4 pF, n=34) could not be determined for all cells. Currents displayed a typically linear IV relationship and reversed at -3.0 ± 0.3 mV (n=46). Average amplitude at -60 mV was -23.7 ± 1.2 nA (n=46). In contrast, Figure 26 E illustrates the standard IV relationship of a non-induced cell, where small, outward, voltage-dependent conductances generally prevail. On average, these currents reverse at -96 mV (n=12), indicating that they are generated by K⁺ efflux from the cell. Interestingly, in the absence of the agonist, outward currents in non-induced cells are notably reduced (Fig. 26 E, grey) and IV curves reverse at more positive potentials, indicating a small non-specific action of ADPR on endogenous HEK cell channels. At -60 mV, this ADPR-generated component was <40 pA in amplitude, which represents a maximum error of about 0.17 % in the recordings from induced cells.

When recording from induced HEK-TRPM2 cells, simultaneous removal of expected permeant cations Na⁺ and Ca²⁺ from the extracellular solution (2.2.5: ES-8) resulted in the complete abolition of the inward component of the ADPR evoked current (97.3 ± 0.3 % of control, n=30), and a 55 mV shift in the reversal towards more hyperpolarized values (Erev = -50.9 ± 1.2 mV, Fig. 26 F-H). Curiously, outward current flow was also depressed but this was minimized with short 30 s applications of NMDG⁺, which additionally allowed an almost
complete recovery upon return to the ordinary recording conditions (89.2 ± 1.7 % of control, n=14).

As illustrated in Figure 26 I-K, perfusion of ACA 20 µM produced a block of the ADPR-induced current (95.3 ± 1.9 % at -60 mv, n=10), but was not as easily washed out (20.8 ± 5.4 % of control, n=10). In this example, the Na⁺- and Ca²⁺-free solution was applied during the wash in order to distinguish between genuine recovery and potential leak. Hereafter, both ACA and NMDG⁺ effects were used repeatedly as a signature of TRPM2.

3.3.3 Heterologously expressed TRPM2 is temperature sensitive
The melastatin related channel TRPM2 is not amongst the emblematic temperature sensors in the TRP superfamily, hence this aspect of its regulation has received relatively little attention. The aim was or the next set of experiments was to enquire whether TRPM2 exhibited some degree of sensitivity to temperatures above 24°C, in analogy to what had been described for I_{ADP} in neurons. Initially, a low concentration of ADPR (5 µM) was included in the intracellular solution, which resulted in no appreciable changes in the whole-cell currents of tetracycline induced cells for periods in excess of 300 s at room temperature (24°C). In 5 out of 8 (60%) cells however, TRPM2-like currents of variable sizes developed after this time. We repeated the experiment but this time increasing bath temperature up to 30 or 36°C in the interval between 125 and 175 s (2-3.5 min) after the start of the recording, in this way allowing for the ADPR solution to be equilibrated inside the small volume of the cell. Temperature elevations to 36 but not 30°C, activated TRPM2 within 29.6 ± 3.6 s (n=12, Fig. 27 A, B) of increasing bath temperature, that is on average 200 s before it normally developed at 24°C. IV relationships of the currents measured
at 36°C were linear (Fig. 27 A, inset) and reversal potential was -9.4 ± 0.8 mV (n=12). ADPR- and temperature- induced currents reached an average maximum of -18.9 ± 4.4 nA at -60 mV and subsequently underwent fast desensitization (decay time = 86.5 ± 16.5 s, n=12, Fig. 27 A, red). For this reason, it was not possible to compare the full size of the current at 36 and 24°C. At 30°C, no currents developed in 400 s of recording, although delayed activation was observed in 1 out of 2 cells where the experiment extended beyond this time. In this cell, the delayed current also desensitized within 100 s, an effect which may reflect a degree of temperature sensitivity even at 30°C, since this phenomenon is not so pronounced at room temperature. In the absence of ADPR, however, steps to 37, 40 and 43°C failed to evoke any current through TRPM2 channels.

Figure 27. Heterologously expressed TRPM2 channels are sensitive to warm temperatures. A) Representative time courses of the changes in HEK-TRPM2 whole-cell currents at 24°C (blue) and when temperature was increased to 30 (orange), 36 (red) or >36°C (brown). Red shaded area represents the interval at which temperature steps were applied. ADPR 5 µM was present in the intracellular solution, except for the higher temperature experiments (>36°C). Inset: example IV of the current activated at 36°C in the presence of ADPR. B) Summary of the data: 24°C n=21, 30°C n=6, 36°C n=12, >36°C n=3.

It is concluded that temperatures around or above 36°C facilitate both the activation and decay of TRPM2 channel currents when low amounts of ADPR
are included in the pipette solution, but are *per se* not sufficient to activate the channel.

### 3.3.4 TRPM2 pharmacology

The next step was to cross-examine some of the pharmacological characteristics known for either $I_{\text{ADP}}$ or TRPM2, such as the effects of SKF96365, 2APB, the agonist cyclic ADPR (cADPR) and AMP.

Both SKF96365 (50 µM) and 2APB (25 µM) exhibited strong inhibitory effects on the HEK-TRPM2 current (Fig. 28 A-C). In relative terms, these effects were slow when compared to that observed in ion substitution experiments (100 and 200 s respectively vs 25 s). Application of SKF96365 was generally deterring
for the recording and, although the effects were observed in 9 out of 9 cells tested, in 67 % of the cases large leak currents prevented the accurate assessment of the full extent of the inhibition. Figure 28 C therefore summarizes only the maximum effect obtained with SKF96365 (87.7 ± 2.4 %, n=9). The actions of 2APB were more reliable, with a 95.1 ± 1.3 % of the control amplitude being suppressed (Fig. 28 D-F, n=8). Only minimal recovery was observed upon washout of either drug (15.0 ± 7.4 and 11.1 ± 2 % respectively).

For completion, it was also tested whether these drugs could similarly modulate the ADPR enhanced current in pyramidal neurons. Figure 29 presents the results obtained with SKF96365 (20 µM). A large inhibition was observed in 3 out of 3 cells tested (amplitude: 87 ± 0.9 %; charge transfer: 91.3 %) together with an apparent increase in the Ca²⁺ influx. This was followed by partial recovery of the boosted I_{ADP} in the normal bath solution: 85.1 ± 6.2 % for amplitude and 68.7 ± 8.1 % for charge. Likewise, application of 2APB at 25 µM respectively blocked 60 and 45 % of control charge and amplitude of the ADPR-enhanced I_{ADP} (n=2). Whilst this may be a gross overestimation of the actual extent of the inhibition given the concomitant changes in series resistance noted, the induced increase in the Ca²⁺ spike and the occurrence of a faster inward current preceeding it (both described secondary responses to this drug when applied on I_{ADP} alone, see section 3.2.1), constitute a clear indication that 2APB affects the ADPR-enhanced I_{ADP}.

Like most TRP channels, TRPM2 can be modulated by many different stimuli and agonists. A comprehensive pharmacological characterization by Kolisek et al. (2005) shed light over other endogenous molecules as potential regulators of this current. These include the oxidant H₂O₂ and the Ca²⁺ release agonist
cADPR. I therefore sought to test some of their actions on heterologously expressed TRPM2, the ADPR-enhanced $I_{ADP}$ and $I_{ADP}$.

Figure 29. SKF96365 blocks the ADPR-boosted $I_{ADP}$ in cultured pyramidal neurons. A) Example $I_{ADP}$ and Ca$^{2+}$ spike traces in the presence of ADPR showing the effects of SKF96365 and the recovery of both currents upon SKF96365 washout. B) Time course of the changes in $I_{ADP}$ charge transfer for the cell in A. C) Pooled data and sample scatter.

The agonist cADPR was applied intracellularly at a concentration of 500 µM in hippocampal neurons, where no significant changes in the time course of the appearance and stabilization of $I_{ADP}$ was observed within almost 15 minutes of recording ($n=6$, Fig. 30 A). When HEK-TRPM2 cells were exposed to this same concentration (and batch) of the drug, large whole-cell currents were established after $364 \pm 97$ s of recordings ($n=8$, Fig. 30 B-D), in marked contrast to the speedy development of currents with ADPR. The voltage dependence of the cADPR-induced TRPM2 currents was linear and currents were readily
suppressed by either the perfusion of ACA 20 µM or NMDG+/Mg2+ (92.2 ± 2.5 % and 95.6 ± 1 % of control, n=4 in each case). Mean amplitude at -60 mV was -31.7 ± 4 nA and the reversal potential 1.3 ± 0.7 mV (n=8).

Figure 30. Actions of cADPR on hippocampal I_{ADP} and heterologously expressed TRPM2 channels. A) Time course of the development of I_{ADP} in control conditions (n=3-9, black) and in the presence of cADPR (500 µM; n=4-6, blue) (p=0.715, two way ANOVA). B) Example of the whole-cell TRPM2 currents recorded in HEK-TRPM2 cells when cADPR 500 µM was added to the intracellular solution. Na⁺- and Ca²⁺- free solution was applied to confirm the absence of leak after ACA application. C) Representative IV traces for the cell in B, illustrating characteristic TRPM2 features in the cADPR gated current. D) Summary diagram for the effects of ACA (n=4) and removal of inward permeant ions (n=4) on the cADPR-evoked TRPM2 current.

AMP, on the other hand, has been reported to exert powerful inhibition of the ADPR gating of the channel at a concentration of 1 mM, arguably by means of direct competition for the same binding site in TRPM2 (Kolisek et al., 2005). It had been previously shown that AMP alone at 5 mM was ineffective, if not
slightly enhancing, in inhibiting $I_{ADP}$ (see 3.2.3), but it remained to be established whether the nucleotide was able to prevent the effects of ADPR in hippocampal neurons. Adding AMP 5 mM to the internal solution together with ADPR made no difference to the boosting of $I_{ADP}$ (Fig. 31 A, B). In fact, currents

![Figure 31. AMP did not inhibit ADPR effects in hippocampal neurons or HEK-TRPM2 cells. A) Run up of $I_{ADP}$ charge density in hippocampal neurons as a function of whole-cell time in ADPR only (black, n= 4-19) and ADPR + AMP at 5 mM (blue, n=7-10) conditions (p=0.805, two way ANOVA). B) Representative traces illustrating the result in A. C) Time course of the induction of TRPM2 currents by ADPR in the absence (black, n=6-14) and presence (blue, n=4-7) of AMP (1 mM) (p=0.234, two way ANOVA). D) Example of the result in C illustrating the characteristics of inward and outward current activation through TRPM2 when AMP was added to the pipette solution. E) IV curves for the experiment in D. F) Summary of the AMP 5 mM data in HEK TRPM2 cells (n=7).](image-url)
might have developed faster, since the ADPR effect was already evident in the first trace of the recording in 4 out of 10 cells. For this reason, the data is presented as absolute charge density and not relative to $I_{ADP}$ at the onset of the experiment. Moreover, the input resistance decrease was in the same range as recorded for ADPR alone (277.9 ± 19.3 vs. 250 ± 20 MΩ, n=9 and 13, p=0.351, unpaired t test). In close correspondence with this result, the HEK-TRPM2 experiment showed a similar lack of effect of AMP (1mM) (Fig. 31 C-E). The speed of full TRPM2 current activation was unchanged when AMP was combined with ADPR (AMP+ADPR 33.6 ± 18.6 s vs ADPR 50.4 ± 12.5 s, p=0.41, Mann-Whitney). Maximal current density at -60 mV was comparable to that observed with ADPR alone (2336.2 ± 201 pA/pF vs. 2398.9 ± 239 pA/pF, n=7 and 20 respectively, p=0.88, unpaired t test), as were other TRPM2 current features such as the reversal potential (-2.9 ± 1 mV) and response to Na⁺- and Ca²⁺- free extracellular solution (95 ± 1.1 % reduction, Fig. 31 D-F).

The results thus far attest that, with the exception of the enhancing effects of cADPR, all of the analyzed biophysical and pharmacological features of $I_{ADP}$ and TRPM2 are in close correspondence. It was therefore hypothesized that functional interference with TRPM2 channel expression in neurons should affect $I_{ADP}$.

### 3.3.5 Design and generation of a TRPM2 channel mutant

In order to test this hypothesis, it was decided to suppress TRPM2 mediated currents using a dominant negative approach. To this end, a mutant TRPM2 subunit was created by exchanging the single, negative amino acid glutamate (E) in the extracellular loop region between S5 and S6 at position 960 for glutamine (Q) by *in-vitro* mutagenesis. TRPM2-E960Q had been demonstrated
to completely ablate currents when expressed in HEK293 cells. Additionally, it
was not possible to record TRPM2-like currents from HEK293 cells upon
expression of concatemeric arrangements of TRPM2- and TRPM2-E960Q
subunits. This was the case independently of whether TRPM2-E960Q was
placed first or second in the concatemer (Xia et al., 2008). The absence of
TRPM2 currents in HEK293 cells transfected with concatamers suggests that
TRPM2-E960Q could function as a dominant negative subunit. It was therefore
hypothesized that also the co-expression of non-concatenated TRPM2 and
TRPM2-E960Q subunits would lead to a suppression of the TRPM2 current in
HEK293 cells. If observed, this would allow the use of TRPM2-E960Q in the
suppression of native TRPM2-mediated currents.

In order to test the above hypothesis it was necessary to clone the TRPM2 from
the FLAG-hTRPM2-pcDNA4-T/O (kindly provided by A. Scharenberg) into the
pcDNA3 vector, where transcription is controlled by a CMV promoter, and to
introduce the E960Q mutation. The strategy for this is presented in Figure 32.

In step 1 hTRPM2-pcDNA4-T/O was digested with HindIII and BamHI and the
1.7kb fragment (Fig. 32, brown) was used to generate FLAG-hTRPM2-H/B-
pcDNA3. In hTRPM2-pcDNA4-T/O the original stop codon had been replaced
by a recognition sequence for KpnI. For this reason, in step 2 the original stop
codon found in TRPM2 was reconstructed and an EcoRI recognition sequence
was introduced by PCR. The PCR was digested with BamHI and EcoRI and the
300 bp fragment (Fig. 32, blue) was cloned into FLAG-hTRPM2-H/B-E-pcDNA3,
thereby generating FLAG-hTRPM2-H/B-B/E-pcDNA3, which was verified by
sequencing. In parallel, the middle 2.6kb BamHI fragment (Fig. 32, green) was
cloned into Bluescript KS+ (II), which was subsequently (Fig. 32, step 4) used to
introduce the point mutation (E960Q) by oligonucleotide directed mutagenesis followed by selection of mutants by DpnI digestion. Sequencing of the 2.6kb BamHI fragment confirmed both the presence of the mutation (Fig. 33 A) and the absence of additional changes. In order to assemble the complete TRPM2-E960Q (Fig. 32, hTRPM2-E960Q-pcDNA3, step 5) the BamHII/BamHI fragment carrying the E960Q mutation was isolated and cloned into FLAG-hTRPM2-H/B-
B/E-pcDNA3. In analogy, the previously obtained 2.6 kb BamHI fragment (Fig. 32, green, step 2) was used to generate the complete FLAG-hTRPM2-pcDNA3 (Fig. 32, step 6) for coexpression experiments with the mutants subunit.

An analytical digest of FLAG-hTRPM2-E960Q-pcDNA3 was performed with various restriction enzymes after preparative DNA isolation to show the integrity of the obtained plasmid DNA (Fig. 33 B).

![Image](image_url)

Figure 33. Sequencing and analytical digest of the mutated plasmid. A: Electropherogram confirming the successful introduction of the point mutation in TRPM2-E960Q. B: DNA agarose gel electrophoresis (1% agarose, 0.5x TBE) of an analytical digest of FLAG-hTRPM2-E960Q-pcDNA3; lane 1: HindIII/SalI (H/SII) 6061, 1299, 1102, 699, 606, 198 bp, lane 2: EcoRI/BamHI (E/B) 7080, 2585, 300 bp, lane 3: Apal (A) 8164, 1393, 408 bp.

### 3.3.6 E960Q and wild type TRPM2 channel expression in HEK 293 cells

The amino acid sequences of the loop region between S5 and S6 of both TRPM2 and TRPM2-E960Q channels are presented in Figure 34 A, along with the general topology of the channel including noteworthy protein domains.

Transfection efficiency in HEK cells was high for both constructs, as judged by GFP fluorescence in the coverslip (~ 50 %, see 2.2.4). Electrophysiological recordings from cells transiently expressing non-mutated TRPM2 channels showed that intracellular ADPR 30 μM was able to sustain large, voltage-independent currents of -5.1 ± 0.8 nA in amplitude at -60 mV, which reversed close to 0 mV (-5.8 ± 0.4 mV, n=25). These, as demonstrated previously for the inducible cell line, were highly sensitive to the removal of Ca²⁺ and Na⁺ from the
Figure 34. TRPM2-E960Q expression has dominant negative effects on TRPM2 currents. A) Predicted membrane topology and main structural domains of TRPM2 channels; amino acid sequence of the pore region of TRPM2 and TRPM2-E960Q transiently expressed in HEK cells. B) Overview of the magnitude of whole-cell currents recorded from HEK cells transfected either with TRPM2, TRPM2-E960Q, pcDNA3 or TRPM2+TRPM2-E960Q. Number of cells measured is specified in brackets. C-F) Representative examples of the ADPR- and/or voltage-induced currents in HEK cells transfected as described for B: time courses (top), corresponding IV curves (bottom).

external solution, responding within milliseconds with a complete abolition of the inward component (99.8 ± 0.8 %, n=25; Fig. 34 B, C). Notably, cell capacitance (6.4 ± 0.4 pF, n=69) and current density (840 ± 145 pA/pF, n=25) were substantially reduced in transiently transfected HEK cells compared to the HEK-TRPM2 cell line (10.2 pF and 2400 pA/pF) (p<0.0001, Mann Whitney test). In direct opposition to these results, cells expressing mutant E960Q subunits did not display TRPM2 current activation in the presence of ADPR (24.4 ± 20 pA at
-60 mV, n=10), exhibiting IV curves akin to those of cells transfected with the pcDNA3 vector only (0.2 ± 19 pA at -60 mV, n=9; Fig. 34 B, D, E). Finally, co-transfection of mutant and non-mutant subunits resulted in a significant suppression of ADPR activated currents (93.8 %, Fig. 34 B, F). As illustrated in the example, residual TRPM2 currents (317 ± 137 pA, n=13) were confirmed as non-leak using the NMDG⁺/Mg²⁺ solution.

3.3.7 TRPM2 downregulation in cultured hippocampal pyramidal neurons

Compelled by the evidence so far, I selected TRPM2-E960Q as a promising alternative to pharmacological interference for the study of the molecular identity of I_{ADP} in cultured neurons.

A neuronal transfection method involving exposure to the lipophilic reagent Lipofectamine (see 2.2.4) was used. In contrast to that observed in HEK cells, this appeared to be rather toxic for the hippocampal neurons (i.e. only 20-30 neurons per coverslip survived the procedure after 24 hours). In percentage terms, however, the transfection yield of hippocampal neurons was approximately equal to that obtained for the transient transfection of HEK293 cells: around 10 green fluorescent pyramidal neurons per coverslip (50 %); glia were never transfected.

Recordings were on average 5 minutes long. One of the most striking consequences of the transfection procedure in these mature cultures was the loss of neuronal processes (Fig. 35 A, left panels). Neurons were able to fire action potentials upon stimulation, but synaptic activity was barely present. Capacitance measurements in GFP-fluorescent and seemingly not transfected neurons (not fluorescent) indicated considerable changes in cell surface area: 72 ± 5.4 pF (n=23) vs. 125 ± 8.9 pF (n=4).
Figure 35. Effects of TRPM2-E960Q overexpression in hippocampal neurons. A) Bright field (left) and fluorescent (right) images of pyramidal neurons in culture subjected to transfection with pcDNA3 only or the TRPM2-E960Q construct in combination with GFP. Scale bar: 55 µM. B) Representative \( I_{\text{ADP}} \) recordings (left) and corresponding \( \text{Ca}^{2+} \) currents (right) for each of the transfection conditions in A. C and D) Summary box plots for changes in current and charge density respectively. Sample number is specified in the graph.

Other properties appeared less affected, although a mild regression in normal developmental markers such as resting membrane potential (-58 ± 1.1 vs -61 ±
2.4 mV) and input resistance (530 ± 48 vs 398 ± 31 MΩ) was detected. As a consequence, transfected and not transfected cells were not directly comparable. Furthermore, qualitative evaluation of the Ca²⁺ spike area pointed to a distinct reduction in voltage-induced Ca²⁺ influx in neurons transfected with either pcDNA3 or TRPM2-E960Q (Fig. 35 B, right panels), with obvious direct implications for I_{ADP}. In spite of this, the results indicate that overexpression of mutant subunits brought about a significant 27 % reduction in I_{ADP} current density (vector: 1.5 ± 0.1 pA/pF, n=11 vs. TRPM2-E960Q: 1.1 ± 0.1 pA/pF, n=12; Fig. 35 C). The change was less apparent for charge transfer and duration: charge transfer 0.3 ± 0.04 vs 0.2 ± 0.04 pA/pF (Fig. 35 D); duration 0.69 ± 0.07 vs 0.64 ± 0.09 s. There were no substantial differences between cells transfected with the vector only and not transfected cells in the same coverslip (p=0.4 and 0.8 for current density and charge, respectively; Mann Whitney test). It is clear that the damage caused by the neuronal transfection procedure compromised to some extent the quantitative assessment of this experiment. However, in combination with the pharmacological characterization provided, the observed partial downregulation of I_{ADP} supports the involvement of TRPM2 channels in the generation of this current in hippocampal neurons.
3.4 ADP-ribose-mediated effects on the activity of CA1 neurons in hippocampal slices

The last part of this study focused on the potential involvement of $I_{ADP}$ in regulating the firing properties of neurons in the CA1 region of the hippocampus. In particular, I was interested in investigating whether this conductance may affect spike frequency adaptation, a role normally ascribed to post spike hyperpolarizations. To this end, I conducted whole-cell patch clamp recordings in acute hippocampal slices of young adult rats in the presence of the $I_{ADP}$ and TRPM2 enhancer ADPR (300 µM). Hippocampal sections were chosen over primary cultures on the basis of their morphological and functional proximity to physiological conditions.

3.4.1 Unmasking $I_{ADP}$ in CA1 pyramidal neurons

In the first instance, it was necessary to confirm the presence of $I_{ADP}$ in hippocampal slices and to establish adequate conditions for subsequent recordings. Neurons ($V_{rest} = -57.3 \pm 0.8$ mV, $n=21$) were voltage-clamped at -60 mV and stimulated with 100 ms-long depolarizing steps to +10/+30 mV. The AHP blockers dTubocurarine and XE991 successfully unmasked $I_{ADP}$ in 21 out of 21 CA1 neurons recorded at 24, 27 and 30°C (Fig. 36). Current amplitude was generally larger than observed in cultured neurons (272 ± 69 pA, 24°C; 352 ± 51 pA, 27°C; 358.3 ± 58 pA, 30°C), as were the associated Ca$^{2+}$ currents. On the other hand, current deactivation was at least three fold faster than in cultures at 24 and 27°C (215 ± 20 ms and 265 ± 59 ms, $p>0.05$, unpaired t test) although a significant increase in decay time was recorded at 30°C (688 ± 143 ms). In connection with this, it should be noted that when experiments were performed at the lower temperatures, a slow outward current developed at the tail of $I_{ADP}$, thereby introducing a possible error in the measurements of the
decay time. Such current was not observed at 30°C. For this reason, as well as for being closer to physiological conditions, the latter was selected as the recording temperature in following experiments.

Figure 36. \(I_{\text{ADP}}\) is revealed upon pharmacological suppression of AHP currents in hippocampal slices. A) Example traces illustrating the characteristics of the \(I_{\text{ADP}}\) unmasked in slice recordings at 30°C after application of dTubocurarine 100 µM and XE991 5 µM. TTX 0.5 µM and TEA 1mM were added to the bath solution at the same time as AHP blockers. The intracellular solution contained 8CPT cAMP 50 µM. HP = -60 mV. B) Summary of \(I_{\text{ADP}}\) amplitude at 24, 27 and 30°C measured in independent experiments (p>0.5, one way ANOVA). C) Decay time of \(I_{\text{ADP}}\) recorded under different temperature conditions (30°C, p<0.05, one way ANOVA).

3.4.2 Effects of ADPR on \(I_{\text{ADP}}\) in hippocampal slices

Before attempting to unmask potential physiological roles of \(I_{\text{ADP}}\) in this preparation I first tested the actions of SKF96365, a confirmed inhibitor of \(I_{\text{ADP}}\), on the medium duration AHP current in primary cultures. It was found that this current, predominantly mediated by SK channels (Stocker et al., 1999), was also suppressed by this TRP channel modulator (Fig. 37), for which reason it was
dismissed as a tool for selective $I_{ADP}$ inhibition. Therefore, in subsequent functional experiments, the influence of the TRPM2 agonist ADPR on voltage and current clamp recordings was evaluated instead.

Figure 37. Action of SKF96365 on $I_{AHP}$. A) Representative $I_{AHP}$ traces and Ca\(^{2+}\) spikes (inset) illustrating the complete abolition of the SK-mediated current and the apparent enhancement of Ca\(^{2+}\) influx upon bath application of the TRP channel inhibitor SKF96365. Recordings were performed at room temperature in the presence of synaptic blockers, 0.5 µM TTX and 1 mM TEA (HP = -50 mV). B) Time course of the experiment in A. C) Summary of the data (p=0.0056, paired t test).

Although the actions of ADPR on $I_{ADP}$ in primary cultured neurons had been already described, it was important to characterize the effects of this drug in a more native environment, given the markedly distinct nature of the two preparations. When ADPR was included in the pipette solution at the same concentration used for experiments with cultured neurons, an enhancement of $I_{ADP}$ was observed (Fig. 38 A-C). This effect was, however, less pronounced and only statistically significant for $I_{ADP}$ charge transfer (-28.4 ± 4 pC vs. -41.7 ± 3.4 pC, n=6 in both cases). A slower and noisier component of $I_{ADP}$ was evident.
under these conditions and current decay time was delayed by at least 1s (1812 ± 173 ms, n=6; Fig. 38 D). In addition, in the presence of ADPR there was a clear tendency to a faster run up of $I_{ADP}$ (Fig. 38 E), with only 1 of 6 cells displaying the opposite behaviour. Thus, the time to reach half maximal charge transfer decreased from 6.9 ± 1.2 to 5.0 ± 0.9 min (n=6; p=0.24, unpaired t test). Overall the effects of ADPR on $I_{ADP}$ in CA1 neurons support the assumption that this conductance is the same as the one characterized in primary cultures.

Next I performed current clamp recordings at 30°C in order to measure the impact of ADPR on the afterpotentials generated following a burst of spikes in the neuron. Spontaneous excitatory synaptic activity was frequently observed at this temperature. Bursts of action potentials were evoked by 400 pA somatic current injections for 400 ms in the absence of all channel blockers or inhibitors. As expected, under control conditions Ca$^{2+}$ influx following a group of spikes consistently triggered an afterhyperpolarization with two distinct kinetic components (see Introduction). A further increase in the hyperpolarization was observed for both the area and amplitude of this potential during the first 20 minutes of whole-cell recording (Fig. 39 A, B and C; black), corresponding to the run up of underlying m$I_{AHP}$ currents. At this point, the average mAHP amplitude was -2.8 ± 0.3 mV (n=12). The peak was reached 72.8 ± 2.9 ms after the end of the current injection. In contrast, the presence of ADPR induced an overall depolarizing shift in the AHP, leading to its progressive decrease and effectively uncovering an overlapping afterdepolarization (Fig. 39 A, B and C blue). This ADP was in fact visible as a positive afterpotential in 30% of the cells recorded. The $I_{ADP}$ enhancer delayed the peak of the AHP (-1.6 ± 0.6 mV, n=11) by approximately 17 ms (89.7 ± 8.6 ms after current step) with respect to control.
Figure 38. Effects of ADPR on \( I_{\text{ADP}} \) in hippocampal slices. A) Representative current traces showing the increased area under \( I_{\text{ADP}} \) in the presence of ADPR. B, C) Summary bar diagrams of the enhancing actions of ADPR on current amplitude and charge transfer (statistics: unpaired t test). D) ADPR significantly prolongs the duration of \( I_{\text{ADP}} \) (\( n=6 \) in both cases; unpaired t test). E) Time courses of the changes in charge transfer in controls and in cells where ADPR was included in the pipette solution (control, ADPR; \( n=3-6 \); \( p<0.0001 \) two way ANOVA).

Figures 39 B and C summarize the change in both peak amplitude and integral of the afterpotential during the first 20 minutes of recording for control and ADPR-treated cells. The effect of ADPR was significant only for the mAHP amplitude, whilst changes in area directly related to drug action may have been masked by the simultaneous activation of the sI\( \text{AHP} \) in these neurons (Fig. 39 D).
Figure 39. ADPR affects the amplitude and area of the afterpotential. A) Representative current clamp traces illustrating the action potential firing (top panels) and following afterpotentials recorded at -60 mV in CA1 neurons upon stimulation with a 400 ms-long current injections of 400 pA in amplitude. Control (black) and ADPR (blue) afterpotential traces are expanded in the bottom panels. The arrows indicate the time points used for the measurement of mAHP and sAHP amplitudes (70 ms and 1 s after the end of the stimulus, respectively) and the shaded box represents the time interval (250 ms from the peak) considered for the calculation of the area under the curve corresponding to the development of the mAHP; this window was the shortest possible to reduce contamination by sAHP components. B, C) Summary of the changes in mAHP peak amplitude and area in the first 20 minutes of recording, illustrating that the presence of ADPR (blue, n=11) was able to counteract the hyperpolarizing shift in the afterpotential normally observed in control conditions (black, n=12). D) Grouped data showing the parallel run-up of the Ca^{2+}-dependent sAHP in both control and ADPR-treated cells. The average change in sAHP was the same in both groups (p=0.568; unpaired t test).

Indeed, when measuring the integral of the afterpotential, the opposing influences of ADPR and the run-up of the slow Ca^{2+}-activated K^+ current could
not be dissociated, even when the area under the curve was calculated using progressively narrower time windows in order to avoid sAHP contamination.

ADPR-treated cells fired on average less action potentials than the control group in response to the 400 ms-long current injections (20 minutes: ADPR 12.9 ± 0.8 spikes, n=12 vs. control 15.6 ± 1 spikes, n=11, p=0.03; unpaired t test). As a result it is possible that the magnitude of the intracellular Ca\(^{2+}\) rise following the burst of spikes was lower with ADPR. Since the SK-mediated \(I_{AHP}\) is Ca\(^{2+}\)-dependent, the depolarizing shift in the afterpotential observed could be attributed to a reduced activation of SK channels in the presence of ADPR. For this reason, it was decided to compare the changes in the afterpotential (as opposed to absolute values) from 0 min to 20 min of whole-cell recording, since within individual experiments the number of spikes did not vary significantly during this time (ADPR cells p=0.76; control cells p=0.46; paired t test). Therefore, the changes observed in the afterpotential may not be directly linked to a reduced Ca\(^{2+}\) influx in ADPR treated cells.

These results illustrate the enhancing effects of ADPR on both \(I_{ADP}\) and the corresponding afterpotential in hippocampal acute slices and support the possibility of a dynamic interaction between AHP and ADP in these neurons. This argument is further strengthened by the following experiment in cultured neurons, where, in the absence of dTC or XE991, the extracellular Na\(^+\) concentration was diminished, so as to reduce the inward component of \(I_{ADP}\). Under these conditions, the outward SK-mediated \(I_{AHP}\) appeared enhanced (Fig. 40). In analogy with the action of ADPR on the area under the curve for the afterpotential, the most pronounced effect of the low Na\(^+\) conditions was on the charge transfer of \(I_{AHP}\) (187.2 ± 24 pC, n=7).
3.4.3 Effects of ADPR on pyramidal neuron excitability

The reduced firing observed in the presence of ADPR could have been related to drug-induced changes in action potential properties in these neurons. To examine this possibility, 5 ms-long current injections of varying amplitude were used to evoke single action potentials. The threshold voltage, calculated 20 minutes after establishing the whole-cell recording, showed a substantial shift towards more depolarized potentials (Fig. 41 A, B) in ADPR-treated cells (-33.8 ± 2.1 mV, n=9) when compared to control (-40.9 ± 1.2 mV, n=6) (p=0.025, unpaired t test). Aside from a raised threshold, action potentials generated in the presence of ADPR did not differ substantially from those induced under control conditions, although there was a tendency towards a broadening of the spike and acceleration of the decay time of the fast \( I_{ADP} \) (Fig. 41 C).
Figure 41. ADPR modulation of action potential properties. A) Single action potentials illustrating the raised firing threshold in ADPR cells. Individual stimulation protocols are shown underneath each trace. B) Mean threshold (filled circles) was statistically different between the two groups. C) Summary of the effects of ADPR on other action potential parameters (statistics: unpaired t tests).

A more detailed evaluation of the firing behaviour in response to somatic current injections of 1 s in duration confirmed a marked reduction in spike frequency at all current injections tested when ADPR was added to the intracellular solution (Fig. 42 A, B). The maximal frequency attainable under these conditions was 19.8 ± 2.6 Hz (n=9) vs. 27.2 ± 3.8 Hz (n=6) for control cells. In a similar manner as demonstrated before in cultured neurons, the TRPM2 enhancer significantly reduced input resistance during the course of the experiment (Fig. 42 C) without affecting the holding current at -60 mV (ΔI_{hold}= -1.2 ± 8.8 pA, control; 16 ± 12.1 pA, ADPR; p=0.17; unpaired t test). Both this effect and the reduction in firing frequency were evident from the moment of achieving the whole-cell configuration. The lowered input resistance implied that ADPR-treated cells required larger current injections than control cells to generate similar numbers of action potentials and, as a consequence, a similar
increase in intracellular Ca$^{2+}$. For this reason, when analyzing the influence of $I_{\text{ADP}}$ on the excitability of the cell, it could be biasing to compare trains of action potentials in response to similar current injections assuming that $I_{\text{ADP}}$ is activated to the same extent by this level of depolarization in cells with and without ADPR. Hence, to approximate equal intracellular Ca$^{2+}$ conditions it was decided to group measurements of trains with a similar number of action potentials, as well as by the magnitude of the current injection.

Figure 42 D shows the reduction in the duration of the second interspike interval (ISI) in a 1s-long train of action potentials observed in ADPR-treated cells when the data was grouped according to the number of spikes fired. This effect, particularly obvious for trains with less than 14 spikes, indicates an underlying increase in excitability in the presence of ADPR, otherwise masked by the robust changes in input resistance and ensuing decrease in firing frequency observed. Additional spike interval analysis revealed a decrease in the instantaneous frequency along the train, thus indicating spike frequency adaptation (SFA) in both control and ADPR cells after 20 minutes of recording (Fig. 42 E). The relative overall drop in instantaneous frequency (i.e. freq. at 1st ISI / freq. at 20$^{\text{th}}$ ISI) appeared unaffected between control and ADPR cells ($p=0.8$; unpaired t test). Despite this, and considering the effects of the ADPR-mediated drop in input resistance already described, it was decided to analyze SFA in more detail to examine potential ADPR- ($I_{\text{ADP}}$) related changes in the first 275 ms of the train.

Figure 43 presents the results obtained with different methods of quantification of early SFA in these neurons. In general, this type of analysis is used to describe the strength of adaptation by comparing the ISI and/or spike number at
Figure 42. ADPR-induced changes in firing behaviour of hippocampal CA1 neurons. A) Superimposed trains of action potentials showing a reduced firing frequency in ADPR-treated cells. Corresponding stimulation protocols are pictured under each trace. B) Mean changes in spike frequency observed under each experimental condition after 20 minutes in the whole-cell configuration (control n=6-12, ADPR n=9-11, p<0.0001, two way ANOVA). C) Characteristic decrease in input resistance observed with ADPR (unpaired t test). D) Comparison between second interspike interval in control and ADPR-treated cells (p=0.03, two way ANOVA). Input resistance drop is compensated by grouping trains with a similar number of action potentials (control n=3-23, ADPR n= 6-18). E) Overall changes in instantaneous firing frequency in the presence and absence of ADPR (control n=3-9, ADPR n=3-10 p<0.0001), two way ANOVA.

the beginning and at the end of the time interval of interest. Thus, a large coefficient of variation of the ISI (CV, Fig. 43 A), a low ratio between action potential number at the end and the beginning of the train (Fend/Finit, Fig. 43 B)
Figure 43. Effects of ADPR on spike frequency adaptation in hippocampal neurons. A-C) Diagrams describing the methodology employed to quantify SFA. D-F) Coefficient of variation, Fend/Finit and last ISI/first ISI graphed as a function of current injection (control: black, n=6-13; ADPR: blue, n=4-9) (G-I) or of the number of spikes in the early stages of the train (first 275 ms) (control: black, n=6-26; ADPR: blue, n=5-23). Statistics: two way ANOVA.

or a high ratio between last and first ISIs (last ISI/first ISI, Fig. 43 C), all denote strong spike accommodation. As explained before, data were grouped according to the magnitude of the current injection or the number of action potentials fired (in this instance, in the early phase of the train corresponding to the first 275 ms). In all cases, there was a positive relationship between the magnitude of the stimulus and the strength of adaptation (Fig. 43 D-F). This was less obvious when the data were averaged according to the number of action potentials fired.
potentials in the early phase of the train (Fig. 43 G-I). Despite varying degrees of sensitivity, all three methods were in agreement with the fact that boosting $I_{\text{ADP}}$ with the TRPM2 enhancer ADPR results in reduced SFA in CA1 neurons. According to CV measurements, the most significant differences were at lower current injections (150-300 pA, Fig. 43 D) or when cells fired up to 12 action potentials (Fig. 43 G). This was consistent for the rest of the methods used, with the exception of the ratio between ISIs, which detected a larger reduction in SFA due to ADPR at current injections above 300 pA.

Finally, it was noted that in the presence of ADPR the depolarizing envelope of the action potential train (Fig. 44 A, B) was significantly augmented. This

Figure 44. ADPR affects action potential amplitude and depolarizing envelope in a spike train. A) Voltage responses to 1s-long current injections of 250 pA in amplitude from varying baseline (depending upon resting potential of the cell) illustrating the increase in the depolarizing envelope towards the end of the train and progressive reduction in action potential amplitude. B,C) Summary of the data: control n=11, ADPR n=8 (unpaired t test).
phenomenon was associated with greater spike amplitude attenuation (Fig. 44 A, C) in these neurons. Frequently, the development of a depolarizing envelope brought about a cessation of firing before the termination of the stimulus. This phenomenon, known as depolarization block, reflects increased inactivation of Na\(^+\) channels and is presumably due to a sustained low-level depolarization during the train, possibly through Ca\(^{2+}\) accumulation. The number of cells exhibiting depolarization block increased from 30 to 50 % in the presence of ADPR. The analysis of spike attenuation included only cells where the last action potential occurred at least 800 ms into the train.

In summary, ADPR 300 µM influenced various aspects of pyramidal neuron physiology. Primarily, the TRPM2 enhancer significantly lowered the input resistance of these cells from early stages of the recording, which had further implications for the normal firing activity of the neuron (i.e. reduced firing frequency and voltage threshold). However, when this effect was compensated for by grouping the data according to the number or action potentials fired in the first 275 ms rather than the magnitude of the stimulus, it became apparent that boosting I_{ADP} actually made neurons more excitable. Similarly, SFA analysis demonstrated that when ADPR was included in the intracellular solution, the balance between coexisting depolarizing and hyperpolarizing afterpotentials consistently shifted towards a larger afterdepolarization, thus decreasing action potential accommodation and favoring depolarization blocks.
Chapter Four

Discussion
The present work centred upon a Ca$^{2+}$-dependent inward current of medium duration initially observed in voltage clamp recordings from the CA1 region of the hippocampus. The elucidation of the molecular correlate(s) of this $I_{\text{ADP}}$ would facilitate the assessment of its potential role in the control of neuronal activity, such as has been suggested for other post spike currents. The question of the molecular identity of $I_{\text{ADP}}$ was approached using a primary culture system, where an in-depth study of the properties of this afterdepolarizing conductance were undertaken. Functional data was gathered from whole-cell recordings in hippocampal slices using a pharmacological tool characterized on the endogenous $I_{\text{ADP}}$ of cultured pyramidal neurons.

4.1 The morphological and electrophysiological characteristics of hippocampal pyramidal neurons are adequately preserved in primary cultures

Dissociated cultures of hippocampal neurons have been used extensively since their introduction in the late 1970s (Banker and Cowan, 1977) in studies ranging from the detailed characterization of synaptic responses and receptor distribution, to cell growth, chemotaxis and excitotoxic neuronal death. Often, the ease of introduction of genetic tools by either direct injections of DNA or simple transfection methods, as well as the suitability for imaging experiments, have proved valuable in the elucidation of many aspects of hippocampal neuronal function.

In electrophysiological terms, primary cultures present some advantages over slice preparations: it is possible to have a much stricter control of the extracellular environment when performing pharmacological experiments, and the space clamp problems encountered in thick brain sections are somewhat reduced, at least in the initial stages of development (Mynlieff, 1997). It is rather difficult, however, to
assess how closely related these two biological systems are with regards to the physical and functional integrity of the cells.

Neuronal morphology is affected by the length of time in culture, as well as the culture substrate (Mynlieff, 1999), and this represents a significant disadvantage over slice preparations, where the morphology and synaptic connectivity of the neurons are largely intact. The morphological types observed in the preparation used were in close resemblance with those reported for postnatal hippocampal cultures (Mynlieff, 1997; 1999). The visual identification of pyramidal neurons proved successful in as much as the passive and active properties of the neurons were in correspondence with previous measurements (Evans et al., 1998; Mynlieff, 1999), and the current of interest could be recorded in over 95% of the neurons patched. The input resistances recorded throughout the days in culture (1200-300 MΩ; DIV1-19) are comparable to the slice preparation (1200-200 MΩ, P1-P24; Cingolani and Pedarzani, unpublished) and the progressive change of the neuronal passive properties, illustrated by parameters such as $V_{\text{rest}}$, $R_{\text{in}}$ and $C_m$, together with the establishment of regular firing and spike adaptation with increasing age of the culture, are indicative of an adequate maturation of the neurons (i.e. increase of cell surface area and higher expression of membrane currents, Mynlieff, 1999), while the large standard deviations reported for passive properties illustrate the inherent variability of neurons developed ex situ.

The expression of Ca$^{2+}$ extrusion mechanisms in developing hippocampal neurons is minimal until around DIV 6/7, when the presence of ATP-ases and Na$^+$/Ca$^{2+}$ antiporters abruptly increases in the membrane of these cells (Kip et al., 2006). This may partly explain the difficulty in obtaining longer-lasting recordings in these young cells, where immature Ca$^{2+}$ handling was probably a major cause
of death, especially as a consequence of the stimulating protocols used aiming at increasing intracellular Ca\(^{2+}\) to elicit the current of interest.

Hippocampal neurons are known to form functional synapses in primary cultures, with a prevalence of excitatory over inhibitory connections (Marks et al., 2000; Banker and Goslin, 2002). These are thought to be essential for the correct development of the principal neurons’ physiological phenotype. However, it also has implications for neuronal survival, as the excess of glutamate released may lead to excitotoxicity. In most cases, synaptic contacts are adequately targeted, but it is not uncommon to find aberrant connections in such an artificial setting (Banker and Goslin, 2002). The high levels of synaptic activity and spontaneous firing observed therefore justify the use of AMPA and NMDA blockers throughout the recordings.

### 4.2 \(i_{\text{ADP}}\) is present in cultured hippocampal neurons

The first question was to test whether \(i_{\text{ADP}}\) was present and retained the same features in tissue culture as in brain slices. The inward tail current recorded following a large depolarizing voltage step and the application of mAHP blockers exhibited kinetic features similar to those of the current unmasked with apamin or dTC in slices (decay in the order of hundreds of milliseconds, Giovannini et al., unpublished observations), and clearly distinct from those reported for other ADP currents in the hippocampus (Caeser et al., 1993; Crepel et al., 1994; Jensen et al., 1996). However, \(i_{\text{ADP}}\) amplitude was substantially attenuated with respect to \(i_{\text{ADP}}\) in slices, which is potentially attributable, amongst other causes, to a lower density of \(i_{\text{ADP}}\) channel(s) or VGCC after enzymatic treatment of the neurons. The decrease in Ca\(^{2+}\) influx was evident in the reduced peak of the unclamped Ca\(^{2+}\) current during the step depolarization in cultured neurons. The absence of
statistically significant variations in current density from DIV 6-19 suggests that the channel(s) underlying $I_{ADP}$ and associated activation mechanisms are expressed from early postnatal stages in these neurons. Additionally, because cultured neurons develop dendritic processes in vitro, the presence of $I_{ADP}$ in early stages of the culture (just after enzymatic dissociation) and the progressive increase in current amplitude following changes in surface area roughly indicate that the distribution of these channels is not strictly somatic.

The ADP current subject of this study is kinetically similar to the mGluR-mediated afterdepolarization recently described by Park et al. (2010) in CA1 neurons, the activation of which markedly shifts the firing behaviour of the neurons. However, the data indicate that $I_{ADP}$ does not require the activation of metabotropic receptors to be elicited (as previously established for the slow ADPs and a medium duration ADP in CA1 neurons, see Introduction), but is always present in hippocampal pyramidal cells upon voltage-induced Ca$^{2+}$ influx and coexists with the voltage activated $I_M$ and the Ca$^{2+}$-activated current $I_{AHP}$. This was demonstrated by the unmasking of $I_{ADP}$ with the corresponding inhibitors XE991 (Wang et al., 1998; Yue and Yaari, 2004) and d-TC (Stocker et al., 1999), which have no reported agonistic actions on glutamate or muscarinic receptors. Additionally, the occurrence of excess neurotransmitter concentrations in the vicinity of the recorded neuron can be clearly excluded, since the continuous superfusion with fresh extracellular medium ensures fast solution exchange in the coverslip.

The results presented further indicate that $I_{ADP}$ does not result from the temporal summation of the persistent Na$^+$ current that normally follows single action potentials and mediates the fast ADP current (Jensen et al., 1996; Azouz et al.,
1996) because $I_{ADP}$ is insensitive to TTX. Thus, this current is generated by a separate molecular entity, which activates in response to more sustained depolarizing stimuli in hippocampal neurons. Depolarizing aftercurrents similar to $I_{ADP}$ have been unmasked by the inhibition of potassium currents by high concentrations of TEA (30-70 mM) or apamin in cortical pyramidal neurons (Kang et al., 1998) and nigral dopaminergic (Ping and Shepard, 1999) and GABAergic neurons (Lee and Tepper, 2007).

4.3 Depolarization-induced intracellular Ca$^{2+}$ elevations activate $I_{ADP}$

In order to further characterize $I_{ADP}$ in cultured neurons, its activation mechanism was investigated. It was found that $I_{ADP}$ is dependent on Ca$^{2+}$ influx through VGCC, as previously shown in slices, where application of extracellular Cd$^{2+}$ ablated the current within a few seconds (Giovannini et al., unpublished observations). The first evidence of Ca$^{2+}$-dependence was the complete abolition of $I_{ADP}$ in an extracellular medium devoid of this ion. Surface charge effects on ion channels (Hille, 2001), resulting from exposure to a Ca$^{2+}$ free solution, were compensated for by addition of Mg$^{2+}$. However, the absence of Ca$^{2+}$ may bring about additional changes in Na$^+$ and K$^+$ channel gating properties (Armstrong and Miller, 1990; Armstrong and Cota, 1991; Su et al., 2001), activation of TRPM7 channels expressed in hippocampal neurons (Wei et al., 2007), and temporary reversal of the electrogenic extrusion mechanisms at the plasma membrane (Kip et al., 2006). The outward current observed in nominal zero Ca$^{2+}$ conditions may represent a combination of these effects and/or the occurrence of an additional, Ca$^{2+}$-independent conductance temporally overlapping with $I_{ADP}$, which would potentially affect the accurate measurement of some of $I_{ADP}$ properties, such as decay time constant and amplitude.
The use of BAPTA to selectively chelate intracellular free Ca^{2+} revealed that \( I_{\text{ADP}} \) activation was in fact dependent on increased intracellular Ca^{2+} and not due to the residual activity of VGCC upon pulse termination. This approach, which has been used before for \( I_{\text{ADP}} \) in slices (Giovannini et al., unpublished observations) and in many occasions to ascertain the Ca^{2+} dependency of other afterdepolarizing neuronal currents (Crépel et al., 1994; Guérineau et al., 1995; Fraser and MacVicar, 1996; Yue et al., 2005), assumes that BAPTA eliminates the source of activation of the channel by reducing the free Ca^{2+} concentration to insignificant levels whilst having no impact on VGCC function. It is possible, however, that the observed enhancement of the unclamped Ca^{2+} current is related to a BAPTA-mediated reduction of the Ca^{2+} dependent inactivation of VGCC. When Ca^{2+} chelators are used quantitatively, they can in principle allow inferences regarding the spatial relationship between Ca^{2+} sources and the targets of a Ca^{2+} signal (Augustine et al., 2003). However, experiments were limited to establishing Ca^{2+} as an activation mechanism for \( I_{\text{ADP}} \) and do not provide further clues as to the potential physical association of Ca^{2+} and \( I_{\text{ADP}} \) channels. Nevertheless, because the peak of \( I_{\text{ADP}} \) occurs within ~50 ms of the termination of the pulse (as opposed to ~500 ms for the \( sI_{\text{AHP}} \) for example; Stocker et al., 2004) it would be reasonable to assume proximity between the two.

The changes in \( I_{\text{ADP}} \) amplitude with higher and longer lasting depolarizing steps mirrored the distinctive voltage-dependent activation of calcium channels (Hille, 2001), increasing with modest depolarization and decreasing along with the electrochemical gradient for Ca^{2+} at very positive potentials. This finding implies that \( I_{\text{ADP}} \) activation is a secondary response to Ca^{2+} influx and complements the results obtained with BAPTA. The fact that \( I_{\text{ADP}} \) amplitude only decreased with stimulation beyond +60 mV hints at a potential involvement of non-perisomatic
Ca\textsuperscript{2+} channels in the activation of I\textsubscript{ADP} (ie. possible dendritic origin of I\textsubscript{ADP}), since it would be expected that Ca\textsuperscript{2+} currents be very small at this potential in well-clamped areas of the membrane, whereas dendritic (and R\textsubscript{s}) filtering of the stimulus might attenuate the voltage step in more distant compartments, thus making local Ca\textsuperscript{2+} influx still available for I\textsubscript{ADP} activation.

4.4 I\textsubscript{ADP} is a mixed cationic current

With regards to the ionic contributions to I\textsubscript{ADP}, the experiments with the non-permeant cation NMDG\textsuperscript{+} to replace extracellular Na\textsuperscript{+} and moderately elevated extracellular K\textsuperscript{+} strongly indicate that I\textsubscript{ADP} is the result of the net inward movement of Na\textsuperscript{+}. NMDG\textsuperscript{+} fully abolished the inward component of I\textsubscript{ADP} and enabled the observation of the Ca\textsuperscript{2+}-dependent outflow of K\textsuperscript{+} presumably through the same channels. These data do not exclude the possibility of Ca\textsuperscript{2+} ions contributing to I\textsubscript{ADP} charge transfer across the membrane. The estimated reversal potential of I\textsubscript{ADP} was \(-0\) mV after correcting for the liquid-junction potential, corresponding with that of a mixed cationic conductance, the sign and size of the current being the result of the relative permeability to and the equilibrium potential of multiple ions. The actual movement of K\textsuperscript{+} and Na\textsuperscript{+} through the I\textsubscript{ADP} channel was corroborated by the positive and negative shifts in the current reversal potential observed under altered ionic conditions (Na\textsuperscript{+} removal: -80 mV shift towards I\textsubscript{K} = -93 mV; 2xK\textsuperscript{+}: +52 mV shift towards I\textsubscript{Na} = +102 mV), which imply that I\textsubscript{ADP} is carried and not otherwise dependent on these ions. The fact that in the complete absence of Na\textsuperscript{+} the reversal of I\textsubscript{ADP} was 13 mV more positive than the predicted equilibrium potential for K\textsuperscript{+} favors the hypothesis that part of I\textsubscript{ADP} is sustained by Ca\textsuperscript{2+} influx. The contribution of Cl\textsuperscript{−} to I\textsubscript{ADP} under our conditions should be effectively null, since the ionic distributions predict a reversal potential for Cl\textsuperscript{−} of -64 mV, very close to the holding potential (-60 mV).
The current voltage relationship of $I_{\text{ADP}}$ established its voltage-independent nature, a feature that is reminiscent of many TRP channels (Ramsey et al., 2006). The manipulation of extracellular $K^+$ proved difficult in this respect, as the IV became non-linear at voltages more depolarized than -40 mV, which coincides with the voltage activation threshold for many $K^+$ channels (Storm, 1990). It is well known that $K^+$ is a key player in the regulation of membrane resistance, as demonstrated by the decrease in $R_{\text{in}}$ recorded when its concentration was raised to 7 mM and this, together with the $Ca^{2+}$-free condition, probably influenced leak and voltage-gated conductances thereby affecting the resulting IV curve.

As a note of caution, it should be emphasized that the reversal potential measurements obtained are an approximation and, although care was taken to isolate $Ca^{2+}$-activated currents by means of current subtraction and selective inhibition of $Na^+$ and $K^+$ currents, the results could be biased by the lack of specific blockers of $I_{\text{ADP}}$ and the use of challenging ionic manipulations. Nevertheless, the combined properties of $Ca^{2+}$-dependent activation and mixed cationic nature add to the arguments that set this novel conductance apart from other $I_{\text{ADP}}$ currents described in hippocampal neurons, which are either activated by voltage (Yue et al., 2005; Metz et al., 2005) or induced by metabotropic receptor activation (Caeser et al., 1993; Crépel et al., 1994; Park et al., 2010) and carried by $Ca^{2+}, Na^+$ or a combination of cations.

4.5 Temperature dependence of $I_{\text{ADP}}$ in cultured neurons

In acute hippocampal slices, the temperature dependence of $I_{\text{ADP}}$ was defined as a biphasic relationship whereby the current increased slowly with temperatures from 23 to $25^\circ C$ and steeply with temperature changes in the physiological range (Giovannini et al., unpublished observations). In agreement with these
observations, in primary cultured neurons \( I_{\text{ADP}} \) also augmented in amplitude with higher temperatures, particularly with steps from 27 to 30\(^{0}\)C. This was not an indirect consequence of increased \( \text{Ca}^{2+} \) influx; on the contrary, the \( \text{Ca}^{2+} \) spike was often reduced at higher temperatures. Indeed, this may have led to the underestimation of the actual effect of temperature changes on \( I_{\text{ADP}} \) and could partly explain the lower \( Q_{10} \) values found for current amplitude in the culture system (\( Q_{10} \) 24-30\(^{0}\)C = 3.4) compared with brain slices (\( Q_{10} \) 26-30\(^{0}\)C = 7.5). On the other hand, because \( I_{\text{ADP}} \) was always recorded using an optimized voltage protocol aimed at maximizing \( \text{Ca}^{2+} \) influx and \( I_{\text{ADP}} \) amplitude at 27\(^{0}\)C, if the overall activation depends on both \( \text{Ca}^{2+} \) influx and temperature in a complex fashion, it is possible that channel activity was already close to saturation at the time of temperature elevations, which would result in the comparatively small amplification of \( I_{\text{ADP}} \) here reported. The acceleration of the deactivation kinetics of \( I_{\text{ADP}} \) may reflect a direct effect of temperature on \( I_{\text{ADP}} \) channel gating kinetics or an indirect effect via changes in membrane properties or in the dynamics of other potential modulators of this conductance such as associated proteins. Alternatively, a faster decay of \( I_{\text{ADP}} \) could be justified by heat-induced changes of the local cytosolic \( \text{Ca}^{2+} \) signals necessary to elicit this conductance, either at the level of \( \text{Ca}^{2+} \) influx through VGCC, as reflected by the apparent reduction in \( \text{Ca}^{2+} \) current during the depolarizing pulses, or of \( \text{Ca}^{2+} \) clearance by pumps and transporters, which might be enhanced at higher temperatures.

Temperature dependence has been quantified for many ion channels (\( Q_{10} = 2-3 \), Hille, 2001) and some transporters (\( Q_{10} = 3.6-4 \), Kimura et al., 1987). Unlike \( I_{\text{ADP}} \), the cationic currents induced by muscarinic and mGluR activation (Fraser and MacVicar, 1996; Crépel et al., 1994) are not temperature dependent, whilst both the activation and inactivation kinetics of the \( \text{Ca}^{2+} \)-dependent slow AHP are
strongly temperature sensitive (Sah, 1996). The physiological relevance of the temperature sensitivity of these coexisting neuronal aftercurrents is not clear. This property of $I_{ADP}$ represents one of its strongest links with the TRP channel family, for which the highest $Q_{10}$ values amongst ion channels are recorded (Dhaka et al., 2006).

4.6 $I_{ADP}$ pharmacology: narrowing down the search for candidates

4.6.1 TRP channels are involved in the generation of $I_{ADP}$

The biophysical properties of $I_{ADP}$ considered so far point at TRP channels as the most suitable molecular counterparts of $I_{ADP}$. This was tested pharmacologically using group of generic compounds, which, despite their limited specificity, exhibit key differences between their actions across the TRP groups (Fig. 3, Introduction). The inferences derived from this pharmacological profiling work are discussed below and summarized in Figure 45, a comparative table matching known characteristics of candidate TRP channels with the phenotype of $I_{ADP}$ in hippocampal neurons in culture and in acute slices.

Flufenamate (FFA) is an anthranillic acid derivative potentially useful in the identification of TRP-mediated events. In this study, FFA induced a relatively delayed inhibition of $I_{ADP}$ accompanied by a reduction of the $Ca^{2+}$ current necessary for $I_{ADP}$ activation. The delay might reflect the time required for FFA to permeate the plasma membrane and act on putative intracellular targets. Alternatively, it could indicate a direct action on the $I_{ADP}$ channel, as reported for heterologously expressed TRPM2 channels, where it exhibits an irreversible open-state dependent antagonistic action (Hill et al., 2004). Although FFA has no reported direct actions on VGCC, it is possible that the inhibitory effect observed on the $Ca^{2+}$ current was due to increased $Ca^{2+}$-dependent inactivation of these channels, since this compound is known to stimulate $Ca^{2+}$ release mechanisms in
<table>
<thead>
<tr>
<th>Ca²⁺ activated</th>
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| Voltage insensitive by rectifying by PKC |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | **Evidence from lcom in slices**

Figure 45. Alignment of the biophysical and pharmacological properties of I<sub>ADP</sub> and candidate TRP channels. Red denotes characteristics directly befitting the profile of I<sub>ADP</sub>.
molluscan neurons and rat hippocampal neurons within a similar time frame (Shaw et al., 1995; Partridge and Valenzuela, 2000).

FFA has been used in the dissection of endogenous TRP-generated events in neurons of the hippocampus (Olah et al., 2009), entorhinal cortex (Zhang et al., 2010), cortex (Haj-Dahmane and Andrade, 1999; Yan et al., 2009) and spinal cord (Gerzanich et al., 2009). Of note, the majority of these studies have used FFA concentrations far greater than the one used in the recordings here presented (100-500 µM vs. 20/10 µM), generally disregarding its lack of specificity. Thus, despite the observed changes on the Ca\(^{2+}\) spike, the possibility that FFA might have a direct inhibitory effect on I\(_{ADP}\) at these lower concentrations cannot be discarded, given the magnitude of the effect observed. The data should, however, be interpreted with caution.

Ruthenium red (RuR) has been used extensively as an inhibitor in the TRP channel context. Under the experimental conditions used, it almost halved I\(_{ADP}\) amplitude and attenuated the Ca\(^{2+}\) current, in a similar manner as observed with FFA. It was deduced that RuR might act on more than one target and its mechanism of action on I\(_{ADP}\) is not clearly established by these results. Indeed, RuR has recognized actions on multiple ion channels including VGCC in neurons and other cells (Tapia and Velasco, 1997; Cibulsky and Sather, 1999).

The use of lanthanum and gadolinium is justified by their generalized blocking action on most TRP channels and the fact that at low concentrations they behave as agonists of TRPC4 and TRPC5 (Plant and Schaefer et al., 2003). The results obtained in dissociated neurons strongly suggest that the primary target of with La\(^{3+}\) and Gd\(^{3+}\) (100 and 10 µM) were VGCC, as demonstrated by the rapid (under 1 min) and complete abolition of the Ca\(^{2+}\) currents. This matches the robust block
of neuronal VGCC subunits observed in HEK cells with nanomolar concentrations of these lanthanides (Beedle et al., 2002; Babich et al., 2007) and prevented the assessment of potential direct actions on $I_{\text{ADP}}$.

SKF96365 is also a broad-spectrum TRP blocker used to identify cationic currents generated by TRPC channels in response to metabotropic receptor activation in sensory (Kress et al., 2008), cortical (Yan et al., 2009), Purkinje (Kim et al., 2003), CA1 (Amaral and Pozzo-Miller, 2007; Wang et al., 2007) and entorhinal cortex neurons (Zhang et al., 2010). At a concentration of 50 µM, the observed effects of SKF96365 on $I_{\text{ADP}}$ were threefold: suppression of $I_{\text{ADP}}$, apparent enhancement of preceding $Ca^{2+}$ currents and subsequent decrease in the peak of the unclamped $Ca^{2+}$ current. The initial increase in the $Ca^{2+}$ current area could be explained by either a direct enhancing effect of SKF96365 on VGCC (which would be in contrast with the current knowledge about SKF96365 properties; Merritt et al., 1990) or a novel blocking effect of the drug on the outward currents that normally counteract $Ca^{2+}$ influx upon depolarization. The delayed decrease in the peak of the $Ca^{2+}$ current is explained by the reported actions of SKF96365 on $Ca^{2+}$ channels (Merritt et al., 1990). Using a lower concentration of SKF96365 (20 µM) prevented the secondary reduction of the $Ca^{2+}$ current and provided a clear indication of a genuine inhibition of the ADP-current. Given the accompanying increase in $Ca^{2+}$ currents, the full extent of $I_{\text{ADP}}$ suppression by SKF96365 might have even been underestimated.

The final generic TRP inhibitor examined was 2APB, a debated antagonist of the inositol triphosphate receptor (Maruyama et al., 1997; Bootman et al., 2002). In analogy with SKF96365, application of this drug resulted in a decrease of $I_{\text{ADP}}$ amplitude and an apparent boosting of the $Ca^{2+}$ current. Additionally, 2APB
induced a fast inward transient current during the depolarizing pulse and slowed down the decay of \( I_{ADP} \). These events and the associated changes in holding current recorded, point to the activation of unidentified ionic currents by 2APB on top of the inhibition of \( I_{ADP} \). The agonistic action of 2APB on some of the TRPV subfamily members might help explain these effects. In spite of its poor specificity, there is no evidence to exclude an actual inhibitory effect of 2APB on \( I_{ADP} \), particularly when \( Ca^{2+} \) influx was simultaneously potentiated. However, like SKF96365, 2APB may not be used as the sole proof of identity of the ADP current, but constitutes a valuable complementary tool.

In summary, aside from the specific considerations already made, the prototypical TRP modulators tested on \( I_{ADP} \) – with particular reference to FFA, SKF96365 and 2APB – validate the hypothesis of TRP channel involvement in the generation of this current. Taken together, the data obtained with these drugs is not sufficient to rule out any particular group of TRP channels unless analyzed in the light of the biophysical properties described for \( I_{ADP} \) (ie. thermosensitive, \( Ca^{2+} \)-activated, voltage-independent, relatively high \( Na^+ \) permeability).

### 4.6.2 A candidate amongst thermosensitive TRPs

Canonical TRPs are primarily \( Ca^{2+} \) channels whose activation is generally coupled to G-protein signaling (Putney \textit{et al.}, 2005) and, until otherwise proven, display no inherent temperature sensitivity. The latter is also true for polycystins, the most distantly related TRP channel subgroup. Although the functional complex TRPP2/PKD1 is postulated to form reticular \( Ca^{2+} \)-activated cation channels (Giamarchi, \textit{et al.}, 2006; Delmas, 2005), it is not known whether the subunits coassemble in hippocampal neurons.

For the above reasons, attention was turned to thermosensitive TRP channels,
represented in the hippocampus by TRPV 1-4 and TRPM 2,4,5. The fact that TRPVs display voltage sensitivity, an overall enhanced permeability to Ca\(^{2+}\) over monovalent cations (Alexander et al., 2008), and in the case the TRPV1 and 2 are activated by noxiously hot temperatures above 42 and 50\(^\circ\)C (Caterina et al., 1997; 1999), essentially opposes the idea that they may mediate \(I_{\text{ADP}}\). However, the possibility exists that channels may behave differently depending on the cell type or conditions where they are expressed.

In favorable comparison with \(I_{\text{ADP}}\), TRPV3 and 4 are activated by warm temperatures in the range of 27-39\(^\circ\)C (Smith et al. 2002; Güler et al., 2002). These channels can be distinguished based on the effects of phorbol esters, which are known to activate TRPV4 (Watanabe et al., 2002; Vriens et al., 2007), but not TRPV3 (Xu et al., 2002), in a PKC independent manner. In cultured neurons, \(I_{\text{ADP}}\) was potentiated by both PDBu and the nonPKC-activating 4\(\alpha\)Phorbol, but the effect was accompanied by an increase in the Ca\(^{2+}\) currents during the depolarizing pre-pulse. The latter was probably due to a phorbol ester-induced reduction in the K\(^+\) current that counteracts Ca\(^{2+}\) influx during the voltage step (Doerner et al., 1988). Nevertheless, the delayed decay of \(I_{\text{ADP}}\) (and ensuing increase in charge transfer) could be due to the concurrent activation of TRPV4 channels, much like the effects observed with the TRPV agonist 2APB. Yet, the complete abolition of \(I_{\text{ADP}}\) upon Na\(^+\) removal in the presence of 4\(\alpha\)Phorbol and the negligible changes in input resistance suggest that the contribution of the TRPV component carried by Ca\(^{2+}\) is rather small, even under maximal agonist stimulation.

The final set of TRP modulators tested was intended to uncover a potential role of the melastatin-related TRP channels in the ADP current. Some of their relevant
features include thermosensitivity (2,4,5), preferential permeation to monovalent cations (2,3,4,5), direct activation by Ca\(^{2+}\) (2,4,5) and lack of voltage-dependence (2,3,6/7). Both spermine and AMP are endogenous compounds extensively used in the characterization of TRPM-specific events in native and heterologous expression systems. At first glance, addition of these chemicals to the intracellular solution produced no appreciable qualitative changes on \(I_{\text{ADP}}\); however, data analysis exposed a delayed inhibitory effect of spermine (1mM) on the run up of \(I_{\text{ADP}}\) and a completely unexpected enhancement in current amplitude with respect to control in the presence of AMP (5mM). There were no detectable alterations in Ca\(^{2+}\) currents.

Spermine is a fast blocker of TRPM 4,5 and 7 with IC\(_{50}\) values between 35 and 60 \(\mu\)M in HEK 293 cells (Nilius \textit{et al.}, 2004a, 2005; Ullrich \textit{et al.}, 2005). Considering this, it is striking that at 1 mM, the effects of Spermine on \(I_{\text{ADP}}\) are slow and incomplete. The drug could have indirectly hindered the run up of \(I_{\text{ADP}}\) by affecting a mechanism that supports channel activation, such as the run-up of Ca\(^{2+}\) currents. Curiously, spermine’s actions were restricted to changes in \(I_{\text{ADP}}\) charge transfer, potentially implicating a pharmacologically distinct molecular player in the generation of the slower component of \(I_{\text{ADP}}\). Amongst the known targets of spermine in the TRPM group, TRPM4 and 5 appear to fulfill the most requirements (but see Figure 45). Spermine has no established actions on TRPM 2 or 3, or indeed on TRPV channels (except TRPV1 where it acts as an agonist at high micromolar concentrations), but given its broad spectrum of action on several types of ion channels (Scott \textit{et al.}, 1993), this would be a reasonable prospect.

As mentioned, the effects of AMP on \(I_{\text{ADP}}\) were also tested. The enhanced \(I_{\text{ADP}}\) amplitude and charge recorded in the presence of high concentrations of AMP (5
mM) add to the evidence against TRPM4 channels, upon which the inhibitory effects of this nucleotide have been clearly established (Nilius et al., 2004a). AMP is also considered a competitive antagonist of TRPM2 (Kolisek et al., 2005; Beck et al., 2006; Lange et al., 2008). However, in agreement with the data obtained, two studies have failed to observe AMP-induced inhibition of TRPM2 currents in neutrophil granulocytes (Heiner et al., 2006) and in cell free patches from Xenopus oocytes (Tóth and Csanády, 2010). Thus, TRPM2 channels appear to be the most likely candidates on the basis of their biophysical (voltage-independent gating, preferential Na⁺ permeation) and pharmacological (block by FFA, AMP-insensitive) resemblance to I_{ADP} (Fig. 45).

This hypothesis was explored with the compounds ACA and ADPR. I_{ADP} suppression by ACA was complete, although the parallel reduction of voltage-gated Ca²⁺ influx again prevented the confirmation of a direct action on I_{ADP}. The results were similar to those obtained with the related compound FFA, where the inhibitory effect was also irreversible. Calcium channel inhibition by ACA has not been previously documented, but it could partly explain the reduction of I_{ADP}, whilst the lack of reversibility has been reported for TRPM2 inhibition by ACA in HEK cells (Togashi et al., 2008).

The most concrete evidence of TRPM2 involvement on I_{ADP} was obtained upon inclusion in the pipette solution of ADPR (300 µM), first characterized as an agonist of TRPM2 in HEK cells by Perraud et al. (2001). Endogenous ADPR-evoked TRPM2 currents have been observed in striatal (Hill et al., 2006) and hippocampal (Olah et al., 2009) neurons. To date, TRPM2 is the only confirmed target of ADPR, though there are two reports that question this assertion (smooth muscle cells: Li et al., 1998; macrophages: Campo et al., 2003). Application of
ADPR in cultured hippocampal neurons induced a large increase in amplitude and charge transfer of $I_{ADP}$. Current traces became characteristically noisy and $I_{ADP}$ did not decay entirely within the interval of acquisition (up to 10 s long). This result is consistent with the main mechanism of action of ADPR upon binding to the NUDT9-H domain, an increase in the opening probability of TRPM2 (Perraud et al., 2001). Moreover, the possibility to resolve discrete single channel events on top of the macroscopic $I_{ADP}$ can be explained on the basis of the large unitary conductance of TRPM2 (60-85 pS; Perraud et al., 2001; Sano et al., 2001; Hill et al., 2006; Du et al., 2009). Importantly, ADPR also affected $R_{in}$ in these neurons, indicating additional channel openings at rest and thus a basal, potentially detrimental influx of Ca$^{2+}$, which probably explains the reduced success rate and shorter duration of the recordings.

In conclusion, the pharmacological profile of $I_{ADP}$ and its selective potentiation by ADPR point at TRPM2 channels as the most likely candidates to underlie $I_{ADP}$. This work provides evidence against the potential participation of other TRP channels in mediating this conductance (Fig. 45), although it is possible that a phorbol ester- and 2APB-sensitive cationic current contributes to the slow decay of $I_{ADP}$. Of course, since ADPR is an agonist, and not a blocker, it was necessary to clarify whether the current potentiated by ADPR is the same as $I_{ADP}$ or if it is a separate conductance activated within the same time domain.

### 4.7 TRPM2 channel activation contributes to $I_{ADP}$ in hippocampal neurons

#### 4.7.1 The ADPR-enhanced current is $I_{ADP}$

In order to ascertain the identity of the ADPR-induced current, its sensitivity to Ca$^{2+}$ and Na$^{+}$ was examined, as hallmarks of $I_{ADP}$. The abrogation of currents by a Na$^{+}$-free, NMDG$^{+}$-based extracellular solution corroborated that Na$^{+}$ is also the
main charge carrier when ADPR is present. Additionally, removing extracellular Ca\(^{2+}\) or augmenting the Ca\(^{2+}\) buffering capacity of neurons by including 10 mM BAPTA in the pipette solution resulted in complete suppression of the ADPR-enhanced current, indicating that, in analogy with I\(_{\text{ADP}}\), this conductance is activated by depolarization-evoked elevations of intracellular Ca\(^{2+}\) and that ADPR alone, at least at this concentration, is unable to activate TRPM2 in hippocampal neurons. This is in disagreement with the general view of Ca\(^{2+}\) as a cofactor of ADPR in native and recombinant TRPM2 channel activation (McHugh et al., 2003; Starkus et al., 2007; Lange et al., 2008). The discrepancy was recently resolved by Du et al. (2009) who demonstrated that Ca\(^{2+}\) alone is sufficient to activate TRPM2 channels expressed in HEK293 cells, likely through a CaM-dependent conformational readjustment of the channel protein (Tong et al., 2006; Du et al., 2009; Csanády and Töröcsik, 2009). Subsequently, a separate group confirmed ADPR-independent, Ca\(^{2+}\)-induced activation of TRPM2 currents in hippocampal pyramidal neurons, as identified by pharmacology and siRNA-mediated downregulation of TRPM2 channels (Olah et al., 2009). Although TRPM2 is often linked to cytotoxicity, its activation by Ca\(^{2+}\) has profound physiological implications, particularly in excitable cells. This said the contribution of endogenous levels of ADPR (main product of CD38 enzyme in these neurons, Ceni et al., 2003) to the generation of I\(_{\text{ADP}}\) in intact neurons should not be discarded.

In order to definitively match TRPM2, I\(_{\text{ADP}}\) and the ADPR-induced current, HEK cells engineered to express TRPM2 channels upon tetracycline treatment were used. Although immunocytochemical analysis revealed FLAG-tagged TRPM2 protein expression in both non-induced (<30% of cells in dish) and induced (>95%) cells, ADPR-evoked activation of whole-cell currents was only observed in the
latter. This suggests that in the absence of tetracycline the expression system is not entirely leak-proof, but the channels are somehow not functional at the plasma membrane. In induced cells, TRPM2 currents evoked by ADPR were similar to those previously characterized (Perraud et al., 2001; Hill et al., 2004; Togashi et al., 2008) and comparable to $I_{\text{ADP}}$ in hippocampal neurons in terms of ionic nature, desensitization and lack of voltage-dependence. The temperature sensitivity of the TRPM2 current in HEK cells constitutes the third report of this feature of TRPM2 channels in heterologous and native expression environments (Togashi et al., 2006; Hermosura et al., 2008). In direct agreement with Togashi et al. (2006), heat stimulation above $36^0\text{C}$ in the presence of ADPR markedly potentiated whole-cell currents indistinguishable from those induced by ADPR alone, speeding up both activation and desensitization of the channels, an effect that resembles the temperature-evoked changes in $\text{Ca}^{2+}$-activated $I_{\text{ADP}}$ amplitude and kinetics.

Finally, pharmacological characterization of heterologous TRPM2 currents was generally consistent with that of $I_{\text{ADP}}$. This work is the first to establish inhibition of this channel by SKF96365 in HEK cells, adding to the expanding list of chemicals that could be used in combination for the dissection of TRPM2-mediated processes. In addition, the data presented confirmed that 2APB exerts a powerful and irreversible inhibition of TRPM2 channels, which remained a controversial issue (Xu et al., 2005; Togashi et al., 2008).

The activation of TRPM2 by alternative ligands such as $\text{H}_2\text{O}_2$ and other adenine nucleotides is also of great interest, because it presents the channel as a proper metabolic sensor. Amongst these agonists, cADPR is reportedly capable to gate the channel either directly ($\text{EC}_{50} = 700 \mu\text{M}$; Kolisek et al., 2005) or in synergy with
factors such as temperature (Togashi et al., 2006). In contrast, others were unable to detect cADPR-stimulated TRPM2 activity (Perraud et al., 2001; Heiner et al., 2006; Tóth and Csanády, 2010). Robust TRPM2 activation by cADPR (500 µM) was observed in HEK-TRPM2 cells after ~350 s of whole-cell recording but no effects were established on the hippocampal \( I_{\text{ADP}} \). The delayed opening of heterologously expressed TRPM2 channels by cADPR has been explained on the basis of residual ADPR contamination of commercial cADPR (Heiner et al., 2006; Tóth and Csanády, 2010). It should be noted that the low amounts of ADPR (5 µM) used in the temperature dependence experiments in these cells also induced late activation of TRPM2 currents (~300 s), suggesting that cADPR-induced currents could have in fact been due to low levels of ADPR contamination of the stock solution used. With regards to the lack of effect of cADPR in hippocampal neurons, it is possible that the concentration of cADPR used (or the ADPR levels therein), was too low to elicit appreciable changes in \( I_{\text{ADP}} \), whereas it was sufficient to gate TRPM2 currents in an over-expressing cell system.

The effects of AMP were more coherent, as the nucleotide was equally ineffective in preventing ADPR-induced currents in hippocampal neurons and HEK-TRPM2 cells at 5 and 1 mM respectively. As a direct product of ADPR hydrolysis, AMP is thought to compete for the binding site in the C-terminus of TRPM2 and inhibit ADPR induced currents with an \( I_{50} \) of 10-70 µM (Kolisek et al., 2005; Lange et al., 2008), but there are differing reports concerning both its ability to suppress TRPM2 currents and to bind NUDT9-H (Heiner et al., 2006; Tóth and Csanády, 2010).

As a whole, the ADPR-enhanced current in hippocampal neurons exhibits characteristics befitting \( I_{\text{ADP}} \), most of which are, in turn, replicated in
heterologously expressed TRPM2 channels. With this notion, a dominant negative strategy for the functional downregulation of TRPM2 channels was designed and implemented in order to validate it as a molecular substrate of $I_{\text{ADP}}$.

4.7.2 Dominant negative suppression of TRPM2 affects $I_{\text{ADP}}$

Loss-of-function mutant ion channel subunits have the potential to selectively interact with native subunits and suppress the activity of multimeric channel complexes. The dominant negative methodology is accurate and fast, which minimizes potential compensatory effects by protein upregulation processes. Dominant negative suppression of TRP channel activity has been previously employed as an effective alternative to siRNA for their silencing (Zhang et al., 2003; Nilius et al., 2005; Chubanov et al., 2007).

Multiple point mutations resulting in non-functional tetrameric TRPM2 channels have been characterized (Mei et al., 2006; Xia et al., 2008). One of these, the substitution of Glutamate for Glutamine in position 960 (TRPM2-E960Q), constitutes a discrete mutation in a sensitive region of the channel protein (pore loop), which does not affect subunit translocation to the plasma membrane but yields channels that do not open in the presence of ADPR (Xia et al., 2008). Recordings from HEK293 cells expressing TRPM2-E960Q confirmed this result. As initially pointed at by Xia et al. using concatemeric expression, the present data indicates that non-concatemeric co-transfection of TRPM2-E960Q and TRPM2 subunits in these cells also produces successful downregulation of TRPM2 currents. Thus, TRPM2-E960Q is a potentially viable tool for the discrimination of endogenous TRPM2 channel function.

The transfection procedure with Lipofectamine2000™ proved relatively stressful for hippocampal neurons when compared to HEK293 cells but did not affect their
electrophysiological integrity. The changes in input resistance and depolarization-evoked Ca$^{2+}$ influx observed in transfected neurons were associated with a loss of neuronal processes and smaller values of membrane capacitance in these cells. Nevertheless, when comparing vector transfected and TRPM2-E960Q transfected neurons (which had undergone the same physical alterations), the dominant negative reduction of $I_{\text{ADP}}$ current density was evident. This result provides direct evidence of the contribution of TRPM2 channels to this current, even if the observed suppression was only partial. In this respect, it is worth stressing that the human and rat TRPM2 channels share 85-88 % identity at the protein level, (Hill et al., 2006; Inamura et al., 2003), which might have reduced the likelihood of interaction between the mutant human and the endogenous rat TRPM2 subunits, effectively producing an incomplete downregulation. Moreover, the interval allowed for protein expression might have been insufficient for the synthesis of adequate quantities of mutant subunits, their transport and assembly at the plasma membrane.

Comprehensive evidence has been provided to support that TRPM2 channel activation in hippocampal pyramidal neurons underlies, at least in part, the generation of $I_{\text{ADP}}$. This conclusion is substantiated by the following facts: TRPM2 forms temperature sensitive, Ca$^{2+}$-activated, non-selective channels expressed at high levels in the principal neurons of the hippocampus; these channels have pharmacological features similar to those of $I_{\text{ADP}}$ (ADPR, FFA, SKF96365, 2APB, AMP) and their functional disruption by dominant negative mutant TRPM2 subunits results in a significant suppression of $I_{\text{ADP}}$ currents.

4.8 Functional implications of $I_{\text{ADP}}$

Having established TRPM2 as a molecular correlate of $I_{\text{ADP}}$ and identified a
potentially selective pharmacological tool to manipulate this conductance, the question of the involvement of $I_{ADP}$ in the activity of hippocampal neurons was addressed. For this purpose, recordings were performed in the CA1 region of acute hippocampal slices, the system where $I_{ADP}$ was first characterized upon selective suppression of afterhyperpolarizing $K^+$ currents. In accordance with previous data, application of sufficient concentrations of dTC and XE991 (100 µM and 5 µM; Stocker et al., 1999; Yue and Yaari, 2004) blocked $I_{AHP}$ and $I_M$ and unmasked a large inward after-current of medium duration. The amplitude and time-related characteristics of the slice $I_{ADP}$, although overall befitting the current recorded in hippocampal cultures, were also different to some extent (i.e. larger and faster), likely reflecting the comparatively preserved nature of Ca$^{2+}$ dynamics in this preparation, where ion channel expression and their interactions with other proteins are intact. The properties of $I_{ADP}$ recorded at different temperatures were mostly in agreement with the results obtained in primary cultures and the acute temperature dependence described by Giovannini et al. (unpublished observations) in that $I_{ADP}$ amplitude increases from room to physiological temperatures. The recordings in brain slices here presented also indicate the occurrence of a smaller conductance with slow kinetics on the tail of $I_{ADP}$, which seems to be also sensitive to temperature. The coexistence of this additional current explains the longer duration of $I_{ADP}$ in slices at warmer temperatures and emphasizes the importance of the use of selective pharmacological tools for the discrimination of $I_{ADP}$-mediated cellular events.

4.8.1 ADPR modulation of $I_{ADP}$: physiological or pathological?

Intracellular application of ADPR (300 µM) indeed enhanced $I_{ADP}$ in hippocampal slices at near physiological temperatures, although its actions on current amplitude and charge were less profound than observed in primary cultured
neurons with the same concentration of the agonist. In analogy with the experiments in cultures, the effect was more significant for the charge transfer, probably due to the increase in open probability of TRPM2. Input resistance was also substantially reduced from the first moments of whole-cell recording, thus affecting the ability of the stimulus to evoke $\text{Ca}^{2+}$ influx in the presence of ADPR. Aside from the saturating influence of temperature and $\text{Ca}^{2+}$ on $I_{\text{ADP}}$ already described, the degree of $I_{\text{ADP}}$ modulation by ADPR in the slice preparation is interesting because it suggests that even at these concentrations, likely exceeding physiological endogenous levels (Heiner et al., 2006; Gasser et al., 2006), damaging $\text{Ca}^{2+}$ influx through TRPM2 channels does not occur. In a study concerning the involvement of TRPM2-mediated $\text{Ca}^{2+}$ rises in ischemia-induced damage in CA1 neurons, Olah et al. (2009) obtained a robust enhancement of $\text{Ca}^{2+}$-activated currents at $30^\circ\text{C}$ in the presence of $1\, \text{mM} \text{ADPR}$, while in cultured hippocampal neurons $300\, \mu\text{M} \text{ADPR}$ was their chosen concentration. This current, evoked by $\text{Ca}^{2+}$ entering the cell during stimulation with voltage-ramps, was ascribed to the selective activation of TRPM2 channels, as it was absent in shRNA-treated neurons in low-density heterogenous cultures and in slices. Altogether, the evidence suggests that, at physiological temperatures, very high concentrations of ADPR are needed to engage pathological activation of TRPM2 channels in hippocampal neurons.

Nevertheless, in pyramidal neurons of the hippocampus ADPR is thought to be the primary product of direct $\text{NAD}^+$ hydrolysis by the inherently active ecto- and endo-enzyme CD38$^+$ (Ceni et al., 2003). Although this and other work (Olah et al., 2009) has demonstrated that ADPR alone is not sufficient to gate TRPM2 currents in hippocampal neurons at concentrations up to $1\, \text{mM}$, endogenous levels of ADPR may predispose the channel for activation by $\text{Ca}^{2+}$, as evidenced by the
faster run up of the whole-cell current observed in the presence of ADPR added to the pipette solution. The synergistic permissive action of ADPR and Ca\(^{2+}\), leading to the activation of TRPM2 channels and \(I_{ADP}\) under physiological conditions, might then influence the firing properties of the neurons.

In other cell types, ADPR and other NAD\(^+\) related metabolites serve diverse physiological roles like the initiation and amplification of immune responses (Sano et al., 2001; Kraft et al., 2004; Heiner et al., 2006; Wehrhahn et al., 2010) or insulin secretion (Togashi et al., 2006; Bari et al. 2009). Recently, two dysfunctional TRPM2 variants have been separately associated with an elevated risk for bipolar disorder (Xu et al., 2009) and a higher incidence of certain neurodegenerative conditions (Hermosura et al., 2008). The pathogenic nature of these mutations, both of which are related to a limited ability of TRPM2 to sustain current flow in the presence of agonists, is strongly indicative of an essential role for these channels in the central nervous system.

### 4.8.2 The interaction between \(I_{AHP}\) and \(I_{ADP}\) underlies the medium duration afterpotential of hippocampal neurons

The properties of voltage- and Ca\(^{2+}\)-elicited afterpotentials were analyzed using 400 ms long somatic depolarizing current injections. The results demonstrate that, even in the absence of TTX, TEA, 8CPTcAMP, dTC and XE991, the moderate enhancing action of ADPR overruled the run up of the mAHP in the first 20 minutes of recording, effectively uncovering the presence of a post-burst ADP. This implied that the underlying \(I_{ADP}\) is present in the absence of pharmacological manipulation of outward currents and can be enhanced by the specific targeting of TRPM2 channels.

Importantly, these experiments also highlight the temporal superimposition of \(I_{AHP}\)
and $I_{\text{ADP}}$. This interplay of inward and outward conductances was characterized in detail for the fast $I_{\text{ADP}}$ and $I_M$ (Yue and Yaari, 2004; Golomb et al., 2006), which follow single or bursts of action potentials, and is also implicit for the $sI_{\text{AHP}}$ and the metabotropic receptor-mediated $sI_{\text{ADP}}$ (Caeser et al., 1993; Guérineau et al., 1995; Congar et al., 1997), present in these and other central neurons. However, this work presents the first evidence of such an interaction between medium duration afterpotentials. Accordingly, in cultured pyramidal neurons, reducing the influx of $\text{Na}^+$ through the $I_{\text{ADP}}$ channels induces a marked increase in the $I_{\text{AHP}}$ charge transfer, which constitutes another argument in favor of a counterbalance between the currents underlying medium duration afterpotentials.

Minor differences in the activation kinetics of $I_{\text{AHP}}$ and $I_{\text{ADP}}$ currents can be inferred from the peak of the membrane hyperpolarization or depolarization observed following an action potential burst (70 ms vs 90 ms after the termination of the stimulus, respectively; also see Fig. 40). Although the mechanism of $\text{Ca}^{2+}$ activation of both SK and TRPM2 is dependent upon CaM binding to the N-terminal portion of each channel subunit (Maylie et al., 2003; Tong et al., 2006), inherent gating features of the channels and/or alterations due to the whole-cell recording mode may be the cause of the observed differences. The association with potential $\text{Ca}^{2+}$ sources, which is very close in the case of SK channels (Marrion and Tavalin, 1998; Bowden et al., 2001), as well as the participation of voltage-dependent KCNQ channels (Tzingounis and Nicoll, 2008) and voltage- and calcium- activated BK channels (Storm, 1989) in the shaping of the afterpotential, should also be considered.

The progressive depolarizing shift in the afterpotential caused by the diffusion of ADPR into the cells and ensuing enhancement of $I_{\text{ADP}}$, was obvious at the peak of
the afterhyperpolarization but not significant when measuring changes in the integral of the afterpotential. It is shown here that the slow activation and decay of the Ca\textsuperscript{2+}-dependent sI\textsubscript{AHP} is a plausible explanation for this apparent lack of effect of ADPR on the integral of the afterpotential. The sI\textsubscript{AHP} exhibits a steady run up during the first 20 minutes of whole-cell recording (Pedarzani et al., 1998). Notably, this K\textsuperscript+ conductance also displays steeply temperature dependent kinetics. As a consequence, sI\textsubscript{AHP} activation is drastically accelerated at 30\textdegree C, rising to peak within 200 ms of the termination of the depolarizing current injection (Lancaster and Adams, 1986), implying that at warm temperatures this current might also partly overlap with I\textsubscript{ADP}, effectively moulding the resulting waveform of the afterpotential. This work raises the possibility that the presence of I\textsubscript{ADP} determines some of the kinetic and temperature-sensitive features of the afterpotential.

A fundamental deduction from the data is that, although at 30\textdegree C all of these conductances together shape the afterpotential, the AHPs normally prevail over the ADP at potentials near rest (e.g. -60 mV). The ADP was observed as a positive afterpotential only in a subset of the cells treated with ADPR (30 %). There are at least two reasons for this: either the diffusion of ADPR into these cells was somehow more effective, or the actual responsiveness of I\textsubscript{ADP} was heightened in these neurons. Curiously, these observations correspond with the low proportion of bursting cells previously reported in microelectrode recordings from CA1 pyramidal neurons (~25 %), which is readily increased upon exposure to 7 mM K\textsuperscript+ (Jensen et al., 1994) or low osmolarity (Azouz et al., 1997) extracellular solutions. Although bursting activity associated with ADPR was not observed in whole-cell recordings from CA1 neurons, it has been previously shown in slices that both high extracellular K\textsuperscript+ and low osmolarity enhance I\textsubscript{ADP}.
(see 3.1.4 and Giovannini et al., unpublished observations). Thus it is tempting to speculate that burst generation in this subset of hippocampal neurons might be partly underpinned by the existence of a highly ADPR-sensitive $I_{\text{ADP}}$, perhaps due to an increased expression of TRPM2 channels.

### 4.8.3 $I_{\text{ADP}}$ is involved in the regulation of neuronal excitability

One of the pitfalls associated with the use of ADPR for the functional identification of $I_{\text{ADP}}$ in neurons was the effect on $R_{\text{in}}$. The reduced firing frequency and elevated action potential threshold recorded are both direct manifestations of the unusually long opening of TRPM2 channels caused by ADPR. The resulting drop in $R_{\text{in}}$ had further practical connotations, because, in order to achieve comparable levels of voltage-evoked intracellular $\text{Ca}^{2+}$ rises (and therefore comparable levels of activation of $I_{\text{ADP}}$) in the presence and absence of the agonist, the magnitude of the stimulus in ADPR-treated cells had to be augmented. Although during data analysis this was compensated for to a certain extent by comparing trains with equivalent numbers of action potentials, TRPM2-mediated $\text{Ca}^{2+}$ (and $\text{Na}^+$) influx at rest was not accounted for and probably added to the differences in $\text{Ca}^{2+}$ levels between the two groups of cells. In fact, the depolarizing influence of this background cationic current could have contributed to the trend towards broader and smaller spikes observed with ADPR, essentially by promoting depolarization-induced $\text{Na}^+$ channel inactivation and counteracting repolarization of the action potential. Also the development of a depolarizing envelope towards the end of the train and ensuing reduction in action potential amplitude are indicative of an underlying accumulation of positive charges on the inner side of the membrane, which eventually resulted in the block of firing with higher stimulus intensity in a high proportion of the cells. Aside from these disadvantages, the main rationale for the use of ADPR was its specificity for TRPM2.
Despite the reduced firing frequencies at different current injections in the presence of ADPR, close examination of the first few inter-spike intervals in 1s-long trains, revealed that ADPR-treated cells actually produced action potentials more readily in response to the same level of depolarization than control cells. In other words, selectively enhancing the TRPM2-mediated afterdepolarization increased the excitability of the neuron. For this reason, considering the widely accepted regulatory role of the medium and slow AHPs on spike frequency adaptation (Stocker et al., 2004) and the newly characterized interplay with the ADP, the functional implications of this ADPR-sensitive component of the afterpotential on the firing pattern of CA1 neurons were analyzed. Once again, the results were consistent with the ADPR-sensitive ADP having a complementary role, together with the mAHP, in setting early spike frequency during 1s-long depolarizations. As demonstrated by three different quantification methods, potentiated TRPM2 channels reduce adaptation in trains with similar numbers of action potentials. Notably, SFA was most affected with lower current injections and fewer action potentials, suggesting differences in Ca\(^{2+}\) sensitivity between $I_{\text{AHP}}$ and $I_{\text{ADP}}$. This means that the permissive role for firing of $I_{\text{ADP}}$ would be more prominent when intracellular Ca\(^{2+}\) rises are modest, for example, during low frequency stimulation, whilst more pronounced elevations would predominantly engage SK channel activity and establish a higher degree of spike accommodation.

The proposed differences in Ca\(^{2+}\) sensitivity of $I_{\text{AHP}}$ and $I_{\text{ADP}}$ may be overcome by the presence of a modulatory agent, whose influence would alter the dynamic balance between these coexisting aftercurrents and ultimately affect the integrative abilities of the neuron. A long-established example of such regulatory activity is the metabotropic receptor-mediated control of excitability. Through
inhibition of sI AHP (Cole and Nicoll, 1983; Knöpfel et al., 1990; Pedarzani and Storm, 1993) and activation of prolonged depolarizing currents (see Introduction), glutamate and acetylcholine exert powerful excitatory actions on pyramidal neurons, which can promote the generation of so-called plateau potentials (Fraser and Mac Vicar, 1996). Similarly, selective block of SK channel activity by apamin has a demonstrated permissive role for firing through depression of the mAHP (Stocker et al., 1999; Oh et al., 2000), an observation that can be re-analyzed in the light of the proposed interplay with TRPM2. The regulation of the phosphorylation state of these SK (Allen et al., 2007) and TRPM2 (Zhang et al., 2007) channels and the redox sensing properties of TRPM2, represent additional mechanisms for the fine-tuning of excitability.

Another important role of SK channels in hippocampal (Ngo-Anh et al., 2005) and cortical (Faber, 2010) neurons is the regulation of synaptic function by hyperpolarizing the membrane in response to glutamate-induced Ca\(^{2+}\) influx, thereby attenuating excitatory synaptic transmission and controlling the induction of plasticity (Lin et al., 2008). In a similar manner, Olah et al. (2009) have recently described functional coupling between TRPM2 channels and NMDA receptors in hippocampal slices, suggesting that SK channels may not be the only players in the loop regulation of synaptic activity. Additionally, TRPM2 channel immunoreactivity was distributed all along the cell body and dendrites of cultured pyramidal neurons, potentially placing them near voltage gated Ca\(^{2+}\) sources in these processes. In this manner, TRPM2 channel activation might contribute to the amplification of travelling signals by locally enhancing depolarization. The recent availability of TRPM2 knockout mice (Yamamoto et al., 2008) should facilitate further investigations regarding the involvement of these Ca\(^{2+}\) gated ion channels in the regulation of somatic excitability and the acquisition of learning
and memory abilities.

In conclusion, two important features of $I_{\text{ADP}}$ (and TRPM2) channels place them at the centre of hippocampal neuron physiology. First, they can sense elevations in intracellular Ca$^{2+}$ by enabling the influx of cations at potentials near rest, depolarizing the membrane for several hundreds of milliseconds. Additionally, the kinetics of $I_{\text{ADP}}$ are closely matched with the activation and deactivation of the $I_{\text{AHP}}$, allowing the concerted regulation of action potential firing frequency both under basal conditions and in the presence of possible modulators. As efficient cellular redox sensors, TRPM2 channels confer unique characteristics to the regulation of the afterpotential, connecting neuronal output to metabolic activity. Although free radicals and excessive degradation of NAD$^+$ could potentially tip the balance in favor of neuronal damage, the regulation of TRPM2 channel activity through Ca$^{2+}$- and temperature-dependent restriction of Ca$^{2+}$ influx, as well as the functional interplay with Ca$^{2+}$- and voltage-sensitive hyperpolarizing currents, are robust mechanisms of cell defense.
Conclusions and perspectives

The present study characterizes a Ca$^{2+}$- and temperature-dependent afterdepolarizing current of medium duration previously unknown for hippocampal pyramidal neurons. $I_{\text{ADP}}$ is unmasked at voltages near rest by pharmacological suppression of afterhyperpolarizing conductances, and is normally present in these cells as demonstrated by selective pharmacological manipulation of the current. The results show that TRPM2 channels mediate at least part of this cationic current, adding to the several other ionic mechanisms known to contribute to the different phases of the afterpotential typically observed in these cells following firing activity. Under basal conditions, $I_{\text{ADP}}$ participates in the regulation of early inter-spike frequency during sustained firing. $I_{\text{ADP}}$ regulation by neuromodulatory transmitters as well as its physiological mechanisms of activation (ie. Ca$^{2+}$ sensitivity and sources, spatial distribution), need to be further investigated. The nature of $I_{\text{ADP}}$ suggests that this current could also be involved in burst firing behaviour and in the generation of plateau potentials in hippocampal neurons, such as has been suggested for other ADP currents. In order to further dissect the functional significance of $I_{\text{ADP}}$ at the network level, future experiments will focus on the identification of specific pharmacological inhibitors of $I_{\text{ADP}}$ (TRPM2). In this respect, it might be possible to synthesise and test compounds chemically related to those that have been shown to inhibit $I_{\text{ADP}}$ and TRPM2 (such as SKF96365, 2APB, ACA or FFA), where simple modifications of their structure may lead to higher specificity of action. Similarly, the implementation of ion channel downregulation strategies suitable for brain slices such as viral delivery of either shRNA specifically targeted at TRPM2 or suitable dominant negative channel subunits, will be at the forefront of future research. Finally, the results
presented emphasize the complexity of the afterpotential as a trait of intrinsic excitability in hippocampal neurons and, in particular, propose a re-evaluation of the characteristics of the mAHP (e.g., mechanisms of generation, ionic basis, pharmacology, temperature dependence) on the basis of the combined profile of SK, KCNQ and TRP channels. This will be greatly facilitated by the development of the above-mentioned $I_{ADP}$ specific tools.
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neuronal death & the function of neural pathways, *Neurochem Int* 30 (2): 137-147


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2APB</td>
<td>2-aminoethoxydiphenylborinate</td>
</tr>
<tr>
<td>8BrcADPR</td>
<td>8-Br-cyclic adenosine diphosphate ribose</td>
</tr>
<tr>
<td>8CPTcAMP</td>
<td>8-(4-Chlorophenylthio)-adenosine-3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>ACA</td>
<td>N-(p-amylcinnamoyl)anthranilic acid</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebro-spinal fluid</td>
</tr>
<tr>
<td>ADP</td>
<td>afterdepolarization, afterdepolarizing</td>
</tr>
<tr>
<td>ADPR</td>
<td>adenosine diphosphate ribose</td>
</tr>
<tr>
<td>AHP</td>
<td>afterhyperpolarization, afterhyperpolarizing</td>
</tr>
<tr>
<td>AM</td>
<td>attachment medium</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>APV</td>
<td>DL-(−)-2-amino-5-phosphonopentanoic acid</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>BDNF</td>
<td>brain derived neurotrophic factor</td>
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<tr>
<td>BPT2</td>
<td>N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl]phenyl]-4-methyl-1,2,3 thia diazole-5-carboxamide</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CA</td>
<td>cornu Ammonis</td>
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<tr>
<td>cADPR</td>
<td>cyclic adenosine diphosphate ribose</td>
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<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>CaMK</td>
<td>calcium/calmodulin dependent kinase</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<tr>
<td>CAN</td>
<td>calcium activated non-selective cation current</td>
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<td>diacylglycerol</td>
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<td>dehydroepiandrosterone</td>
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<tr>
<td>DIV</td>
<td>day in vitro</td>
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<td>DM</td>
<td>dissection medium</td>
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<td>DMEM</td>
<td>Dubelcco’s Modified Eagle Medium</td>
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<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxyribonucleotide triphosphate</td>
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<tr>
<td>DPBA</td>
<td>diphenylboronic anhydride</td>
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<td>DPHTF</td>
<td>diphenyltetrahydrofuran</td>
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<td>Abbreviation</td>
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<tr>
<td>dTC</td>
<td>d-Tubocurarine</td>
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<td>ethylenediaminetetraacetic acid</td>
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<td>EGFP</td>
<td>enhanced green fluorescent protein</td>
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<tr>
<td>fADP</td>
<td>fast afterdepolarization</td>
</tr>
<tr>
<td>fAHP</td>
<td>fast afterhyperpolarization</td>
</tr>
<tr>
<td>FFA</td>
<td>flufenamate, flufenamic acid</td>
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<tr>
<td>GM</td>
<td>growth medium</td>
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<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>HBSS</td>
<td>Hank’s Buffered Salt Solution</td>
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<td>HCN</td>
<td>hyperpolarization-activated cyclic nucleotide gated</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HP</td>
<td>holding potential</td>
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<td>IF</td>
<td>immunofluorescence</td>
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<td>IMS</td>
<td>industrial methylated spirits</td>
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<td>ISH</td>
<td>in situ hybridization</td>
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<tr>
<td>ISI</td>
<td>inter-spike interval</td>
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<td>IPTG</td>
<td>deoxyribonucleotide triphosphate</td>
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<td>KBR7943</td>
<td>2-[2-[4-(4-Nitrobenzyloxy)phenyl]ethy]lisothiourea mesylate</td>
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<td>LB</td>
<td>luria broth</td>
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<tr>
<td>mADP</td>
<td>medium afterdepolarization</td>
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<tr>
<td>mAHP</td>
<td>medium afterhyperpolarization</td>
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<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>mGluR</td>
<td>metabotropic glutamate receptor</td>
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<tr>
<td>NAADP</td>
<td>Nicotinic acid adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotine amide dinucleotide</td>
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<tr>
<td>NB</td>
<td>Northern blot</td>
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<td>NBQX</td>
<td>2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo(f)quinoxaline-7-sulfonamide</td>
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<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
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<tr>
<td>NMDG</td>
<td>N-methyl D-glucamine</td>
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<tr>
<td>OAADPR</td>
<td>O- acetyl-ADP-ribose</td>
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<tr>
<td>OAG</td>
<td>1-oleoyl-2-acetyl-sn-glycerol</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PDBu</td>
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<td>PDDHV</td>
<td>Phorbol 12,13-didecanoate 20-homovanillate</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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</table>
PIP₂  phosphatidylinositol 4,5-biphosphate
PKA  protein kinase A
PKC  protein kinase C
PLC  phospholipase C
PMA  phorbol-12-myristate-13-acetate
RNA  ribonucleic acid
RuR  ruthenium red
RT  room temperature
RT-PCR  reverse transcription polymerase chain reaction
sADP  slow afterdepolarization
sAHP  slow afterhyperpolarization
SB366791  4'-Chloro-3-methoxycinnamalide
SB452533  N-(2-Bromophenyl)-N'([2-[ethyl(3-methylphenyl)amino]ethyl]-urea
SEM  standard error of the mean
SFA  spike frequency adaptation
siRNA  small interfering ribonucleic acid
SKF96365  1-{-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole
TAE  Tris-Acetate-EDTA buffer
TBE  Tris-borate-EDTA buffer
TEA  tetraethylammonium
Tet  tetracycline
TM  transmembrane
TRP  transient receptor potential
TTX  tetrodotoxin
UV  ultraviolet
VGCC  voltage-gated calcium channels
WB  Western blot
XE991  10,10-bis(4-Pyridinylmethyl)-9(10H) -anthracenone dihydrochloride
X-Gal  bromo-chloro-indolyl-galactopyranoside
XIP  exchange inhibitory peptide
ZD7288  4-(N-ethyl-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride