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Use of Patch Clamp Electrophysiology to Identify Off-Target Effects of Clinically Used Drugs

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1. Introduction

Most drugs have effects attributed to actions at sites other than those that are intended. In many cases these off-target effects have adverse consequences, though in some instances these effects may be neutral or even beneficial. Many off-target effects involve either direct or indirect actions on ion channels. Hence, electrophysiological approaches can be employed to screen drugs for effects on ion channels and thereby predict their off-target actions. The pharmaceutical industry routinely uses cellular expression systems and cloned channels to quickly screen thousands of compounds to eliminate those that have well known adverse ion channel effects, such as inhibition of the Kv11.1 potassium channel encoded by the human *Ether-à-go-go* related gene (hERG). However, these methods are not well-suited to predicting many other off-target effects mediated by actions on ion channels natively expressed in specific tissues. We have employed a more directed electrophysiological approach to evaluate a small number of compounds (e.g. drugs with known or predicted adverse effects) to identify ion channel targets that might explain their actions. This chapter will describe this approach in some detail and illustrate its use with some specific examples.

2. Approach

Our general approach to evaluating ion channel effects of a specific drug on a particular cell type involves the following steps:

2.1 Establish an adequate single cell physiological model to evaluate the drug of interest

While expression systems such as the human embryonic kidney (HEK) cell line or Chinese hamster ovary (CHO) cell line over-expressing individual ion channels are useful tools for initial drug screening they may not adequately reflect the functional roles of ion channels in their native tissues. Immortalized or primary cell culture models that retain expression of the same ion channels that are natively expressed in the tissue under investigation should be considered. Some examples include neonatal cardiomyocytes for studying cardiac ion channel function (Markandeya *et al.*, 2011), rat superior cervical ganglion (SCG) neurons for natively expressed neuronal ion channels (Kim *et al.*, 2011; Zaika *et al.*, 2011), and embryonic

rat aortic (A7r5 and A10) cell lines for investigating vascular smooth muscle electrophysiology (Roullet *et al.*, 1997; Brueggemann *et al.*, 2005; Brueggemann *et al.*, 2007). The advantages of using cultured cells compared with freshly dissociated cells from the native tissue include their accessibility, ease of maintenance, high experimental reproducibility, and susceptibility to molecular interventions. However, there are also disadvantages in the use of cultured cells. In particular, the expression pattern of ion channels, receptors, and signaling proteins may differ between cultured cells and native tissues due to differences in proliferative phenotype, absence of surrounding tissues in cell culture and developmental stage from which the cells were derived. Hence, the results obtained using cultured cells should be interpreted with caution and, whenever possible, supplemented by studies performed on freshly dispersed cells and/or by functional assays using intact tissues or live animals.

2.2 Select the patch-clamp mode (e.g. ruptured or perforated patch) for electrophysiological recording

Selection of the patch-clamp mode of recording is generally based on the known properties of the ion channels to be studied. Important considerations include their regulation by phosphatidylinositol 4,5-bisphosphate (PIP_2) and soluble second messengers. The open state of many types of ion channels is known to be stabilized by membrane PIP_2 (Hilgemann & Ball, 1996; Loussouarn *et al.*, 2003; Zhang *et al.*, 2003; Bian & McDonald, 2007; Rodriguez *et al.*, 2010; Suh *et al.*, 2010). With conventional (ruptured) patch-clamp recording, the levels of PIP_2 decrease over time, which can cause irreversible rundown of the currents. Inclusion of Mg-ATP in the internal solution may slow rundown of the PIP_2-dependent currents in excised or ruptured patch recordings (Ribalet *et al.*, 2000), but only the use of the perforated patch configuration enables extended recording of stable whole cell currents for tens of minutes. Regulation of channels via the actions of soluble second messengers may be altered in the ruptured patch configuration as cytosolic solutes may be lost by dialysis into the relatively large volume of the pipette solution. Use of the perforated patch configuration prevents dialysis of signaling molecules and loss of PIP_2 from the membrane. But the ruptured patch-clamp configuration is technically less demanding and so is often preferable if signaling mechanisms are not a concern or if the channels of interest are less dependent on PIP_2 for their activity. Ruptured patch techniques are commonly used for recording currents from voltage-gated sodium channels, Ca_v3 (T type) calcium channels, potassium channels of Kv1, Kv2, Kv3 and K2P families, cystic fibrosis transmembrane receptor (CFTR)-type chloride channels, TRPC family of non-selective cationic channels and ORAI1 store-operated channels. The ruptured patch mode also enables faster data collection, saving the investigator the extra 15-30 min required for patch perforation in each experiment.

The choice of pore-forming agent used for patch perforation is often a matter of personal preference. Pores formed by amphotericin B and nystatin in the membrane under the patch are selectively permeable to monovalent ions (such as K^+, Na^+, Cs^+, Cl^-) preserving cytosolic Ca^{2+} and Mg^{2+} concentrations and all soluble cytosolic signaling molecules (Horn & Marty, 1988; Rae *et al.*, 1991). Use of gramicidin for patch perforation also preserves intracellular Cl^- concentration as gramicidin pores are impermeable to Cl^- (Ebihara *et al.*, 1995). It is possible to record stable currents for several hours in perforated-patch mode from a single cell when appropriate pipette and bath solution compositions are used with continuous bath perfusion.

When using the perforated-patch recording technique, attention should be paid to the value of access resistance achieved. The value of the access resistance (or series resistance as it is also known) will likely exceed pipette resistance by 2- to 5-fold and when current amplitudes are in the nanoampere range will introduce significant error into the true membrane voltage-clamped value. The amount of voltage error can be estimated by multiplying series resistance by current amplitude. If the error exceeds a few millivolts, series resistance compensation should be used.

2.3 Determine the appropriate composition of the internal (pipette) and external (bath) solutions

After choosing a physiological cell model that mimics as closely as possible cells in the intact tissue, it is logical to use intracellular and extracellular solutions with compositions similar to body fluids, at least for initial drug testing. Recipes for different extracellular physiological saline solutions (PSS) such as Krebs-Henseleit solution, Hank's balanced salt solution (HBSS), artificial cerebral spinal fluid (CSF) are readily available in the relevant scientific literature. The pH of the external solution is typically 7.3-7.4.

The composition of the internal (pipette) solution should also closely match known cytosolic ionic composition: high in K^+ (usually in the range of 135-140 mM), low in Na^+ (normally from 0-5 mM). The concentration of Cl^- may vary depending on cell type. For many cultured cells, such as A7r5 cells, stable recordings are most easily obtained using relatively low $[Cl^-]_{in}$, in the range of 30-45 mM, in combination with large impermeable anions such as gluconate or aspartate to balance K^+. For other cell types, including freshly dispersed smooth muscle cells, pipette solutions with 135-140 mM KCl are preferable. If the internal solution will be used in perforated patch-clamp mode, inclusion of Mg^{2+} and buffering of cytosolic Ca^{2+} is not required, as amphotericin and nystatin pores are impermeable to Ca^{2+} and Mg^{2+} (Horn & Marty, 1988; Rae *et al.*, 1991).

For ruptured patch recording, free Mg^{2+} concentration should be set within the range of 1-2 mM and free Ca^{2+} concentration should be approximately 100 nM. To accomplish this, Ca^{2+} buffers such as EGTA, EDTA, or BAPTA should be included in the pipette solution. Free Ca^{2+} concentration will depend on the concentration of the buffers (usually 0.1-10 mM), their binding constants for Ca^{2+} and Mg^{2+} and the amounts of added Ca^{2+} and Mg^{2+}. MAXCHELATOR is a series of programs freely available online (http://maxchelator.stanford.edu/) that can be used for determining the free Ca^{2+} concentration in the presence of Ca^{2+} buffers. Mg- or Na-ATP (1-5 mM) should also be included in the internal solution for ruptured patch recording. The range of pH for internal solutions may vary from 7.2-7.4, usually buffered with HEPES (1-10 mM).

Attention should be paid to the osmolality of both internal and external solutions. Osmolality of body fluids is approximately 275-290 mOsM; the osmolality of the internal and external solutions should be measured with an osmometer, adjusted to the physiological range, and balanced (within 1 or 2 mOsM) between internal and external solutions.

Use of approximately physiological external and internal solutions for patch-clamp experiments enables recording of a mix of ionic conductances for initial evaluation of drug effects on the cell type under investigation.

2.4 Use appropriate voltage clamp protocols (e.g. voltage steps or ramps) to record drug effects on total currents

Design of the voltage protocol should be based on biophysical properties of the ion channels expressed in the cells under investigation. It is very useful for initial drug screening to select a holding voltage close to the resting membrane voltage measured or reported for that particular cell type. A voltage protocol designed to apply a family of long test voltage steps (1-5 s) in both negative and positive directions from the resting membrane voltage allows the investigator to record a mix of both rapidly and slowly activating/inactivating currents through voltage-dependent and voltage-independent ion channels.

The time between voltage steps should be sufficient for channel deactivation.

The stability of the measured currents should be established before testing the effects of drugs on the currents. For example, applying the same series of voltage steps should generate approximately equal currents on successive trials in the same cell. Voltage-ramp protocols can also be used to record instantaneous (voltage-independent) or rapidly activating currents; the ramp can be applied at regular intervals to monitor the stability of the currents over time. For slowly activating currents, use of a single voltage step applied at regular intervals is generally more appropriate for time course measurements.

When stable recordings of total ionic conductances are achieved, it is possible to test the effects of a drug, usually applied at varying concentrations. Drugs may affect the amplitudes of the conductances and the kinetics of their responses to the applied voltage protocols as well as their voltage-dependence of activation. The drug effects are generally time-dependent and vary with drug dose in a reproducible manner. Careful evaluation of the drug effects on total membrane currents provides important clues to the types of ionic conductances that may be affected. It is then desirable to record the drug-targeted ionic conductances in isolation.

2.5 Adjust recording conditions to isolate drug-sensitive currents

To record specific currents among the mix of total cellular ionic conductances, a tailored voltage protocol should be used in combination with internal and external solutions and pharmacological approaches that are rationally chosen to enhance or maintain the current of interest while minimizing other conductances. The voltage protocol should reflect the specific biophysical properties of the channels under investigation. If the data are available, consider the voltage dependence of activation and time constants of activation, inactivation and deactivation of the currents. For example, store-operated currents are known to be inwardly-rectifying and highly Ca^{2+}-selective, with fast Ca^{2+}-dependent inactivation at negative voltages (Parekh & Putney, 2005). To isolate the highly Ca^{2+}-selective store-operated currents from other conductances, consider using an external solution containing 10-20 mM Ca^{2+} and replacing all monovalent ions (K^+, Na^+ and Cl^-) with impermeant ions such as N-methyl D-glucamine and aspartate. A voltage protocol comprised of a 0 mV holding voltage with 100 ms ramps from +100 to -100 mV can be applied every 5-20 s to record the time course of current activation in response to store depletion (often induced by dialyzing cells with EGTA- or BAPTA-containing pipette solution in ruptured patch mode or by application of thapsigargin or cyclopiazonic acid, which block the ability of the cells to sequester Ca^{2+} in the endoplasmic/sarcoplasmic reticulum (Brueggemann *et al.*, 2006)).

In general, the isolation of broad classes of ionic conductances (i.e. Ca^{2+} currents, K^+ currents, nonselective cation currents, or Cl^- currents) may be achieved by using bath and pipette solutions containing ions that cannot permeate or that block the movement of ions through other classes of ion channels. For example, to record Ca^{2+} conductance in isolation, Cs^+ can be used to replace K^+ because it blocks most if not all K^+ channels and thereby minimizes contributions of outward K^+ currents to the recording. Similarly, replacing Cl^- with aspartate, gluconate or sulfonate will minimize contributions of Cl^- conductances.

It is much more difficult to isolate ion currents within the same class. This may require the use of pharmacological agents that are selective for a particular class of channels. For example, if it is desired to isolated T-type Ca^{2+} current from L-type Ca^{2+} current, specific L-type Ca^{2+} channel blockers like verapamil can be used. In this case, an alternative (or adjunct) approach is the use of the ruptured patch mode, which leads to rundown of L-type Ca^{2+} current over time; other Ca^{2+} conductances (e.g. T-type Ca^{2+} currents) that have less tendency to run-down in the ruptured patch configuration, may then be recorded in isolation. The use of pharmacological ion channel blockers to eliminate unwanted conductances should be employed with caution unless the specificity of the drugs has been thoroughly established.

Probably most difficult is the isolation of specific K^+ currents because many different potassium channels are normally expressed in each cell. Several highly specific toxins are available for certain subfamilies of potassium channels (hongotoxin and margatoxin for Kv1.1, Kv1.2, Kv1.3 (Koschak *et al.*, 1998), hanatoxin for Kv2 (Swartz & MacKinnon, 1995), K-dendrotoxin for homo- and heteromeric channels containing Kv1.1 (Robertson *et al.*, 1996)). These can be used to eliminate a subtype of K^+ current or determine the contribution of that subtype to the larger mix of K^+ currents. It is important to consider that different members within a subfamily of K^+ channels can combine to form functional heteromeric channels, which may vary in their sensitivities to toxins depending on the subunit composition (Tytgat *et al.*, 1995; Plane *et al.*, 2005) In some cases, a combination of pharmacological approaches and voltage protocols that take advantage of the unique biophysical properties of the K^+ channels expressed in the cell type under investigation can effectively isolate a specific subtype of K^+ conductance (see example below).

Recording a specific current 'in isolation' from other currents is never fully achieved, but conditions may be established that provide a reasonable signal to noise ratio to evaluate contributions of a subset of ion channels. Specific pharmacological ion channel blockers or activators may be useful to confirm that the currents measured are largely attributable to a particular type of channel, but molecular knockdown approaches are often the best way to determine what fraction of the currents measured are mediated by a specific channel subtype. When conditions have been optimized for recording isolated currents, the effects of the drug on those currents can be tested.

2.6 Evaluate the actions of the drug of interest at its physiologically or clinically relevant concentrations

An appropriate dose-response range of the drug of interest should be based on consideration of physiological or clinically achieved plasma concentrations and doses used in vitro from previously published studies. Dose-dependent effects can be evaluated both

under physiological ionic conditions as well as under recording conditions that isolate specific currents. Stable recording of currents in the absence of drug should be established by applying voltage steps or ramps at regular intervals and measuring similar current amplitudes for several minutes. Increasing concentrations of the drug are then applied, starting at a dose that has little or no effect and increasing in 10-fold or smaller increments to at least the maximum clinical or physiological drug concentration. Be aware that repetitive drug administration or incrementally increasing doses may induce tachyphylaxis. Applying a single dose acutely to a naïve cell may provide the best assessment of the effect of that dose of the drug.

To evaluate whether the presence of the drug changes the biophysical properties of the channel, such as its gating kinetics or voltage-dependence of activation, specific voltage protocols may be applied when steady-state effects of a particular dose of the drug have been achieved. For example, a tail current voltage protocol can be used to evaluate the effects of a drug on voltage-dependence of channel activation. This protocol should be applied at the end of the control recording (before drug application); two successive voltage protocols that yield similar currents establish the stability of the control recording. The same successive voltage protocols should then be repeated when measurement of the time course of current amplitude indicates that the current amplitude has reached a new plateau in the presence of the drug.

To determine the reversibility of drug effects, it is important to measure the currents during drug application and during washout of the drug. It may require tens of minutes to achieve a stable reversal of a drug effect and in some cases the effects will not be reversed within a practical time frame. Reversibility, when it is achieved, provides convincing evidence that the effect measured was specifically due to the drug and not simply due to time-dependent changes such as run-up or run-down of currents. It is also important to include vehicle and time controls to assure that effects are due to the presence of the drug rather than the time of recording or the solvent in which the drug is dissolved.

Reproducible effects of a drug on the amplitude or biophysical characteristics of a particular current in the cultured cell model may provide important clues to the drug's effect on a particular tissue. However, whenever possible, results based on cultured cells should be confirmed using freshly isolated cells from the tissue from which the cultured cells were derived. The electrophysiological characteristics of the drug-sensitive currents may suggest one or more specific ion channel subtypes as the drug targets. Molecular biological approaches may then be used to confirm the identity of the drug-sensitive ion channel.

2.7 Apply molecular biological approaches such as knock-down and overexpression as necessary to confirm an ion channel drug target

As was noted above, cultured cells are often suitable for molecular biological interventions. Knock-down of expression of specific ion channels may be achieved by treatment with short hairpin RNA (shRNA) or small interfering RNA (siRNA). Alternatively, expression of dominant-negative ion channel subunits may specifically abrogate the function of particular ion channels. These molecular constructs can be introduced into the cultured cells using transfection techniques with appropriate plasmids or by infecting the cells with viral vectors engineered to express the constructs. Inactive constructs (e.g. scrambled shRNA) should be

used as a control. Biochemical techniques such as RT-PCR or Western blotting and/or immunohistochemistry are required to confirm the effectiveness of knock-down.

Knock-down of expression or function of a specific ion channel can reveal how much that channel type contributes to the currents measured and whether a drug effect can be attributed to specific actions on that channel type. In electrophysiological recordings, knock-down of a specific channel type should eliminate the contribution of those channels to the currents measured. In other functional assays, loss of the drug effect when the channel is knocked down would provide evidence that the functional effects of the drug can be specifically attributed to its actions on that channel type. Alternatively, if the effects persist even after knockdown of a particular channel then that particular channel is unlikely to be the primary drug target.

Another way to implicate an ion channel as a drug target is to over-express the ion channel and test the effects of the drug on the currents. This is best done in the same cellular environment known to express that type of ion channel endogenously because the cellular environment dictates many properties of ion channels, including regulation by signaling pathways that may at times mediate or modulate drug effects. Overexpression typically results in much larger currents that can be unambiguously attributed to the overexpressed channels. If these channels are direct or indirect targets for the drug, then drug application should have effects on the currents similar to the effects observed on native currents.

Potential pitfalls of these molecular biological strategies include changes in expression or function of other molecules that may compensate for the increased or decreased channel expression or otherwise alter the measured currents. It is important to keep in mind that the effects of drugs on ion currents may not be via a direct interaction with the channel itself, but instead mediated by other mechanisms, including activation of cellular signaling molecules whose expression may or may not be altered when channel expression levels change.

2.8 Establish a multicellular functional system or animal model for final proof of principle

To determine the physiological significance of drug effects on particular types of ion channels, the drug can be tested on in vitro (ex vivo) and/or in vivo functional models. Examples of in vitro functional models include the isolated Langendorff heart preparation (Skrzypiec-Spring *et al.*, 2007), muscle strips of various origins, aortic or bronchial rings, brain slices, lung slices, pressurized artery preparations, etc. These more complex experimental systems more closely mimic physiological conditions, but also introduce additional factors that may complicate interpretation of drug effects. It is important to consider whether changes in tissue function in the presence of a drug can be attributed primarily to the drug's effects on ion channels in a particular cell type. There may be multiple effects on multiple cell types within the tissue.

In vivo drug testing adds a further level of complexity, but it is the ultimate test of how a drug will affect whole animal physiology. Many different animal models have been developed and are described in the literature. To determine whether an effect of a drug in vivo can be attributed to its actions on a specific ion channel, it may be possible to compare

its effects with the effects of another drug that is known to have the same or opposite effects on that ion channel.

3. Example: Cyclooxygenase-2 inhibitor effects on vascular smooth muscle ion channels

The following example illustrates how we have employed the approaches described above in an attempt to elucidate the mechanisms underlying differential adverse cardiovascular risk profiles among clinically used drugs of the same class. Selective cyclooxygenase-2 (COX-2) inhibitors, such as celecoxib (Celebrex®), rofecoxib (Vioxx®), and diclofenac, are non-steroidal anti-inflammatory drugs (NSAIDs) commonly used for the treatment of both acute and chronic pain. About five years after celecoxib and rofecoxib were approved for use in the United States, rofecoxib (Vioxx®) was voluntarily withdrawn from the market because of adverse cardiovascular side effects (Dajani & Islam, 2008). The ensuing investigation of the cardiovascular side effects of this drug class revealed differential risk profiles, with celecoxib being relatively safe, compared with rofecoxib and diclofenac (Cho *et al.*, 2003; Hermann *et al.*, 2003; Aw *et al.*, 2005; Hinz *et al.*, 2006; Dajani & Islam, 2008). Early reports suggested that these differences might relate to pro-hypertensive effects of COX-2 inhibition (Cho *et al.*, 2003; Hermann *et al.*, 2003; Aw *et al.*, 2005; Hinz *et al.*, 2006) that were offset by vasodilatory effects of celecoxib (Widlansky *et al.*, 2003; Klein *et al.*, 2007). However, the mechanisms underlying the vasodilatory effects of celecoxib remained elusive.

We employed the following strategies to investigate whether celecoxib might exert its vasodilatory actions via effects on ion channels in vascular smooth muscle cells (VSMCs). Additional details of these studies were published previously (Brueggemann *et al.*, 2009).

3.1 Vascular smooth muscle cell model

The embryonic rat aortic A7r5 cell line was chosen as the cell model to compare the effects of celecoxib on vascular smooth muscle ion channels with those of rofecoxib and diclofenac. The immortalized A7r5 cell line retains a differentiated smooth muscle phenotype including expression of L-type and T-type Ca^{2+} channels (Qar *et al.*, 1988; Brueggemann *et al.*, 2005), several types of potassium channels (Kv7.5 (Brueggemann *et al.*, 2007), K(Ca)3.1, K(Ca)1.1 (Si *et al.*, 2006) and Kv1.2 (Byron & Lucchesi, 2002)), several members of TRPC family of non-selective cation channels (TRPC6, TRPC4, TRPC1, TRPC7 (Soboloff *et al.*, 2005; Brueggemann *et al.*, 2006; Maruyama *et al.*, 2006)) as well as several Gq-coupled receptors (e.g. V1a vasopressin receptors (Thibonnier *et al.*, 1991), 5-HT2 serotonin receptors (Weintraub *et al.*, 1994), and ET_A endothelin-1 receptors (Bucher & Taeger, 2002). A7r5 cells proliferate in cell culture until they form a confluent monolayer of the cells that are electrically coupled by gap junctions formed by connexins (Cxs) 40 and 43 (Beyer *et al.*, 1992).

3.2 Patch clamp mode

To investigate the vasodilator actions of celecoxib, it was important to consider two types of ion channels that are perhaps the most important in determining the contractile state of vascular smooth muscle cells: Kv7 channels that determine the resting membrane voltage

(Mackie & Byron, 2008), and L-type voltage-gated Ca^{2+} channels, activation of which induces Ca^{2+} influx, smooth muscle contraction, and vasoconstriction (Jackson, 2000). Both of these types of channels are known to be regulated by PIP_2 (Suh & Hille, 2008; Suh *et al.*, 2010). We therefore chose to use the perforated patch-clamp configuration to record currents in voltage-clamp mode (200 μg/ml amphotericin B in internal solution was used for membrane patch perforation).

3.3 Internal and external solutions

For the initial test of celecoxib, rofecoxib and diclofenac, total currents were recorded in A7r5 cells under approximately physiological ionic conditions. The standard bath solution contained (in mM): 5 KCl, 130 NaCl, 10 HEPES, 2 $CaCl_2$, 1.2 $MgCl_2$, 5 D-glucose, pH 7.3. Standard internal (pipette) solution contained (in mM): 110 K gluconate, 30 KCl, 5 HEPES, 1 K_2EGTA, 2 Na_2ATP, pH 7.2. Osmolality was adjusted to 268-271 mOsm/l with D-glucose.

3.4 Voltage clamp protocols

We used a 5s voltage step protocol from -74 mV holding potential to test voltages ranging from -94- +36 mV. After each test pulse the voltage was returned to -74 mV for 10 s to allow full deactivation before the next voltage step was applied. This protocol enabled us to simultaneously record the current-voltage (I-V) relationship for L-type Ca^{2+} channels (based on peak inward currents recorded at the beginning of the voltage steps; see inset on Fig. 1A) and for Kv7 channels (based on steady-state outward K^+ currents recorded at the end of the voltage steps). The evaluation of L-type currents at the beginning of the voltage steps was only possible because of the absence of rapidly-activating K^+ currents in the voltage range used. The long (5s) voltage steps enabled relative isolation of Kv7 currents at the end of the voltage steps because Kv7 channels do not inactivate, whereas most other K^+ channels do inactivate when stepped to a constant activating voltage for 5 s. Representative current traces and I-V relationships are shown on Fig. 1A and 1B.

The I-V voltage protocol requires approximately 4 min to complete all the 5 s voltage steps with a 10 s interval between each step. We repeated this three times to determine that the currents were stable (the I-V curves were approximately superimposable). When the currents were stable we initiated a voltage protocol designed to record the time course of drug application. The time course voltage protocol combined 100 ms voltage ramps (from a -74 mV holding potential to +36 mV) to record the rapidly-activating Ca^{2+} current (as the peak inward current) followed by 5 s voltage steps to -20 mV to record slowly-activating and non-inactivating Kv7 current (measured as the average steady-state current recorded at the end of the voltage step; Figure 1C). The time course protocol was applied every 15 s. Ca^{2+} and K^+ currents were recorded for at least 5 min before application of celecoxib (10 μM). Celecoxib was then applied until a stable drug effect was achieved (approximately 15 min). Then the I-V voltage-step protocol was applied again (twice in succession) to record I-V relationships of the Ca^{2+} and K^+ channels in the presence of the drug. The time course protocol was then re-initiated to monitor the effects of washout of celecoxib. These experiments revealed that celecoxib induced a reversible enhancement of Kv7 current and inhibition of L-type Ca^{2+} current—both of these effects could potentially contribute to the vasodilatory actions of celecoxib.

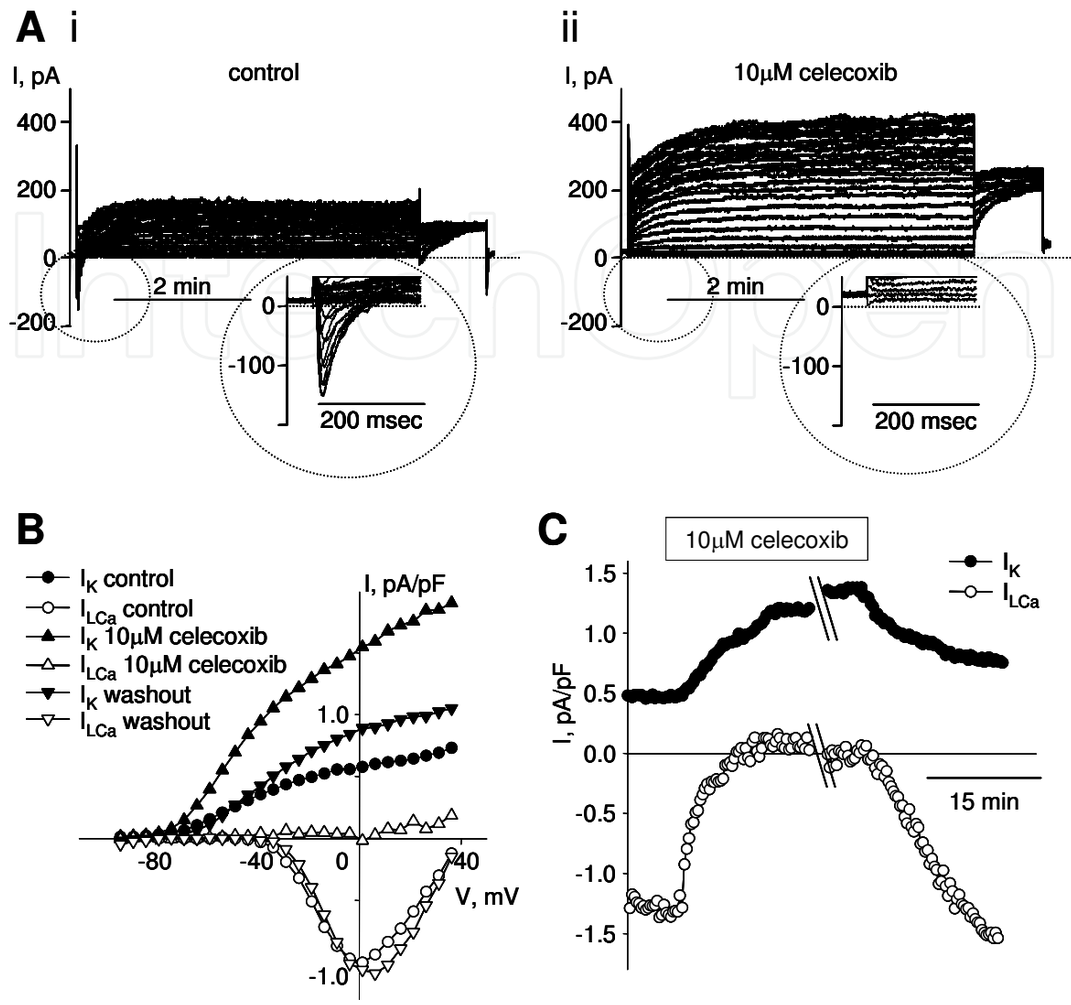

Fig. 1. Enhancement of K^+ current and inhibition of Ca^{2+} current by celecoxib in A7r5 cells.

A, representative traces of whole-cell K^+ and Ca^{2+} currents measured in a single A7r5 cell; i, control; ii, in the presence of 10 μM celecoxib. Inward Ca^{2+} currents, activated at the beginning of the voltage steps, are shown in insets on an expanded scale for clarity. B, I-V curves, corresponding to traces in A, for steady-state K^+ current (filled symbols) and peak inward Ca^{2+} current (open symbols) in control (circles), in the presence of 10 μM celecoxib (triangles), and after washout of celecoxib (inverted triangles). C, corresponding time course of inhibition of the peak inward Ca^{2+} current and activation of K^+ current. Reproduced with permission from Brueggemann *et al.*, (2009).

Similar experiments were conducted to evaluate the effects of other NSAIDs (rofecoxib and diclofenac), 2,5-dimethylcelecoxib (a celecoxib analog lacking COX-2 inhibitory activity), as well as verapamil (a known inhibitor of L-type Ca^{2+} channels) and flupirtine (a known activator of Kv7.2- Kv7.5 channels) (Brueggemann *et al.*, 2009). The effects of these drugs were compared with celecoxib-induced effects on L-type Ca^{2+} currents and Kv7 currents. From these studies it was apparent that neither rofecoxib nor diclofenac mimicked celecoxib in its actions on either L-type Ca^{2+} currents or Kv7 currents; on the

other hand, 2,5-dimethylcelecoxib was indistinguishable from celecoxib in its effects (Brueggemann *et al.*, 2009).

3.5 Isolation of L-type Ca^{2+} currents and Kv7 currents

To evaluate in more detail the actions of celecoxib on L-type Ca^{2+} currents and Kv7 currents, each type of current was recorded in isolation. To record Ca^{2+} currents in isolation, a Cs^{+}-containing internal solution was used (for A7r5 cells, the internal solution contained (in mM): 110 Cs aspartate, 30 CsCl, 5 HEPES, 1 Cs-EGTA, pH 7.2). Isolated Ca^{2+} currents were recorded with a 300 ms voltage step protocol from -90 mV holding potential. To isolate Kv7 currents, 100 μM $GdCl_3$, sufficient to block L- and T-type Ca^{2+} channels and non-selective cation channels, was added to the external solution. Isolated Kv7 currents were recorded with the same 5s voltage-step protocol used to record a mix of currents (see above).

3.6 Effects of celecoxib at therapeutic concentrations

Celecoxib dose-response curves for L-type Ca^{2+} currents and Kv7 currents were obtained by measuring the currents during successive applications of increasing concentrations of celecoxib (ranging from 0.1 μM to 30 μM), each time waiting until the drug effect had stabilized before application of the next dose. The celecoxib concentrations selected for dose-response determinations were based on a ± 10-fold range of mean therapeutic concentrations typically achieved in the plasma of patients treated with celecoxib (1-3 μM) (Hinz *et al.*, 2006). Our estimated IC_{50} value for suppression of L-type Ca^{2+} currents was 8.3 ± 1.3 μM (Brueggemann *et al.*, 2009).

To extend the findings to a more physiological model system, the effects of celecoxib were also examined using freshly dispersed mesenteric artery myocytes. Celecoxib inhibited Ca^{2+} currents and enhanced Kv7 currents recorded in isolation in mesenteric artery myocytes, just as had been observed in A7r5 cells (Brueggemann *et al.*, 2009).

3.7 Molecular biological approaches to evaluate Kv7.5 as a target of celecoxib

Kv7 currents measured in A7r5 cells had previously been attributed to Kv7.5 (KCNQ5) channel activity based on expression studies and on elimination of the currents by shRNA treatment targeting the Kv7.5 (KCNQ5) mRNA transcripts (Brueggemann *et al.*, 2007; Mani *et al.*, 2009). To determine whether Kv7.5 was a specific target for celecoxib, we measured the effects of celecoxib on overexpressed human Kv7.5 channels, using the A7r5 cells as an expression system. Celecoxib robustly enhanced the overexpressed Kv7.5 currents (Brueggemann *et al.*, 2009).

3.8 Functional assays to evaluate how ion channel targeting by celecoxib affects cell and tissue physiology

As noted above, Kv7 channel activity is believed to stabilize negative resting membrane voltages in arterial myocytes and thereby opposes the activation of L-type voltage-gated Ca^{2+} channels. The latter mediate Ca^{2+} influx, smooth muscle contraction, and vasoconstriction. Drugs that enhance Kv7 channel activity or that directly inhibit L-type Ca^{2+} activity would therefore be expected to reduce cytosolic Ca^{2+} concentration, relax the

arterial myocytes, and dilate arteries. To test the hypothesis that the effects of celecoxib on arterial smooth muscle ion channels contributes to its vasodilatory actions, three different functional assays were used:

a. Arginine-vasopressin (AVP) is a vasoconstrictor hormone that has been shown to induce Ca^{2+} oscillations in confluent monolayers of A7r5 cells. We therefore loaded A7r5 cells with the fluorescent Ca^{2+} indicator fura-2 and examined the effects of celecoxib (10 μM) in comparison with rofecoxib (10 μM) on AVP-induced Ca^{2+} oscillations. In support of our hypothesis, celecoxib opposed the actions of the vasoconstrictor hormone, essentially abolishing AVP-stimulated Ca^{2+} oscillations. Rofecoxib, in contrast, had no effect (Figure 2A). Inhibition of AVP-stimulated Ca^{2+} oscillations was also observed using known L-type Ca^{2+} channel blockers or activators of Kv7 channels (not shown, but see (Byron, 1996; Brueggemann *et al.*, 2007)).

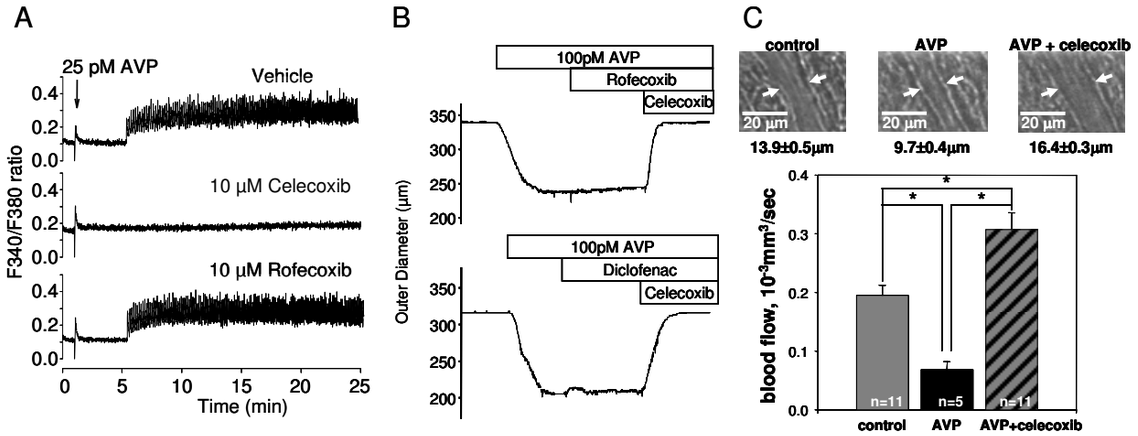

Fig. 2. Functional assays to evaluate celecoxib ion channel actions.

A, Celecoxib, but not rofecoxib, abolishes AVP-induced Ca^{2+} oscillations in A7r5 cells. Confluent monolayers of fura-2-loaded A7r5 cells were treated with 25 pM AVP (arrow). Representative traces show the absence of AVP-induced Ca^{2+} oscillations with simultaneous addition of celecoxib (10 μM, middle) but not with addition of vehicle (top) or rofecoxib (10 μM, bottom). B, representative traces from rat mesenteric artery pressure myography illustrating the inability of 20 μM rofecoxib (top) and 20 μM diclofenac (bottom) to dilate arteries preconstricted with 100 pM AVP. Celecoxib (20 μM) fully dilated the same arteries when added after either rofecoxib or diclofenac. C, measurement of arteriolar blood flow in vivo using intravital microscopy reveals that AVP (100 pM) significantly constricts arterioles (top panels) and reduces blood flow (bar graph), but this effect is more than fully reversed by the addition of 10 μM celecoxib. Panels A and B reproduced with permission from Brueggemann *et al.*, (2009).

b. The constriction and dilation of arteries can be measured in vitro using pressure myography. Small segments of artery are cannulated at either end, pressurized to their normal physiological pressure, and maintained at physiological temperatures and ionic balance; arterial diameter is monitored continuously by digital image analysis while drugs are applied to the bath. We used these methods to test the ability of celecoxib to dilate pressurized mesenteric artery segments that were pre-constricted with AVP (100

pM). In support of our hypothesis, celecoxib induced concentration-dependent, endothelium-independent dilation of pre-constricted mesenteric arteries. Similar effects were obtained using known L-type Ca^{2+} channel blockers or activators of Kv7 channels (not shown, but see (Henderson & Byron, 2007; Mackie *et al.*, 2008)). The maximum dilatory effect of celecoxib was achieved at a concentration of 20 μM; neither rofecoxib nor diclofenac induced significant artery dilation at the same concentration (Figure 2B).

c. Finally, it is important to evaluate the effects of the drug in an in vivo model. We therefore examined the ability of celecoxib to increase blood flow in mesenteric arterioles of live anesthetized rats using intravital microscopy and intravenous perfusion of fluorescent microspheres. AVP (100 pM) superfused over the exposed portion of the mesenteric vasculature induced significant arteriolar constriction and reduced blood flow (determined from the velocity of fluorescent microspheres moving through the arterioles). Application of celecoxib (10 μM) in addition to AVP more than fully restored both arteriolar diameter and blood flow (Figure 2C).

The combined functional assays provided strong evidence supporting the hypothesis that celecoxib, but not other NSAIDs of the same class, exerts vasodilatory effects via combined activation of Kv7 potassium channels and inhibition of L-type voltage-gated Ca^{2+} channels in arterial smooth muscle cells. These results may explain the differential risk of adverse cardiovascular events in patients taking these different NSAIDs.

4. Conclusion

Carefully designed and executed electrophysiological experiments can provide important insights into the mechanisms of drug actions, including their off-target effects on specific tissues.

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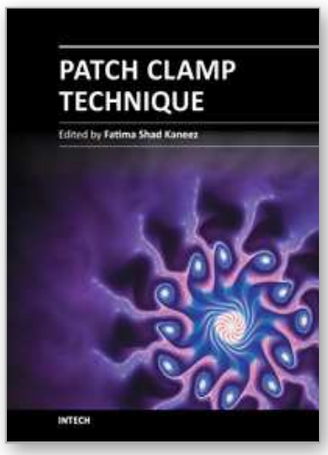

Patch Clamp Technique
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This book is a stimulating and interesting addition to the collected works on Patch clamp technique. Patch Clamping is an electrophysiological technique, which measures the electric current generated by a living cell, due to the movement of ions through the protein channels present in the cell membrane. The technique was developed by two German scientists, Erwin Neher and Bert Sakmann, who received the Nobel Prize in 1991 in Physiology for this innovative work. Patch clamp technique is used for measuring drug effect against a series of diseases and to find out the mechanism of diseases in animals and plants. It is also most useful in finding out the structure function activities of compounds and drugs, and most leading pharmaceutical companies used this technique for their drugs before bringing them for clinical trial. This book deals with the understanding of endogenous mechanisms of cells and their receptors as well as advantages of using this technique. It covers the basic principles and preparation types and also deals with the latest developments in the traditional patch clamp technique. Some chapters in this book take the technique to a next level of modulation and novel approach. This book will be of good value for students of physiology, neuroscience, cell biology and biophysics.

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