

TRP channels as drug targets

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Abstract. Ca^{2+} channel antagonists acting on electrically-excitabile cells have proved to be valuable therapeutic agents. The discovery of such agents and the identification of their molecular target resulted from the investigation of unexpected actions of known pharmacological agents. Ca^{2+} influx through receptor-operated channels in electrically non-excitabile cells such as leukocytes is also functionally important, but to date the channels involved have not been successfully exploited as drug targets for anti-inflammatory therapy. Until recently, research in this area has been hindered by the lack of obvious molecular identity, but the emergence of the transient receptor potential (TRP) cation family has yielded promising candidates which may underpin the different receptor-operated Ca^{2+} influx pathways present in leukocytes. In addition, receptor-operated Ca^{2+} influx channels are also expressed in electrically-excitabile cells suggesting that receptor-operated Ca^{2+} entry pathways are likely to be of wider significance and emphasizes the breadth of their potential as novel, and as yet, unexplored and unexploited drug targets.

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Ca^{2+} influx through plasma membrane channels in all cell types is of fundamental importance in both physiology and pathophysiology, e.g. contraction of muscle cells, neurotransmitter release from nerve terminals, secretion by epithelial cells and leukocyte activation. Therefore modulation of cell function by targeting these channels represents a potentially effective approach for therapeutic intervention.

The two main Ca^{2+} influx pathways which have been under investigation are those Ca^{2+} channels which are voltage-activated and those which are not. Although their function is similar — they regulate the source of one of the most important cytosolic signalling messengers in the body, Ca^{2+} — the drug discovery path trodden by researchers is distinctly different.

Discovery of L-type voltage-operated Ca^{2+} channel blockers

Blockers of L-type voltage-gated Ca^{2+} channels (L-VOCCs) present in vascular smooth muscle are widely used and very effective drugs for the treatment of

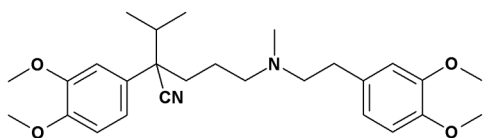
cardiovascular diseases such as hypertension and, in particular, angina. The very earliest compounds, verapamil and prenylamine (Fig 1), from Knoll and Hoechst respectively, were developed as coronary vasodilators working as β -adrenoceptor antagonists. However, both had unexpected cardio-suppressant side effects, which Fleckenstein investigated, and he discovered by chance that they were in fact Ca^{2+} channel blockers (Fleckenstein 1983). He demonstrated that these compounds mimicked the cardiac effect of withdrawal of Ca^{2+} ions, which inhibits the cardiac excitation-coupling resulting in diminished contractile force. These effects could be rapidly overcome by addition of Ca^{2+} ions, β catecholamines or cardiac glycosides. Hence the initial concept of Ca^{2+} antagonism was coined disproving the initial assumption that both of these compounds were β -adrenoceptor antagonists (Fleckenstein 1983). More Ca^{2+} antagonists were subsequently identified such as D600, a methoxy derivative of verapamil (Fig. 1), which had a similar profile to verapamil although it was more potent, and in 1969, the dihydropyridines nifedipine and niludipine (Fig. 1) which were also strong coronary vasodilators and had negative inotropic effects showed a similar mechanism to verapamil and D600. These observations led Fleckenstein to propose a new distinct pharmacological class of potent inhibitors of contraction-excitation coupling existed, the ' Ca^{2+} antagonists'. The biochemical isolation and identification for the site of action of the compounds came from the availability of highly radioactive ^3H -labelled nitrendipine (Fig. 1) which demonstrated high affinity binding to protein extracts from heart, coronary arteries and aorta leading to the identification of the α subunit of the L-VOCC and subsequently the additional accessory subunits of the native channel. The results of these biochemical experiments also suggested that the dihydropyridines, which are chemically different Ca^{2+} antagonists to verapamil or diltiazem (Fig. 1), did not have identical binding sites on the channel nor shared a common mechanism of action, e.g. state-dependence of inhibition by dihydropyridines. The differential binding affinities of the dihydropyridines measured in a range of tissues also supported existence of different channel subtypes in different tissues.

Molecular cloning of the L-VOCC subunits came in the early 1990s and functional data obtained with these cloned proteins confirmed the biochemical, pharmacological and electrophysiological characteristics of the native channel.

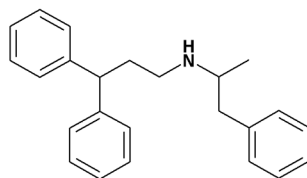
Hence, the unexpected effect of pharmacological agents in functional cardiovascular assays led to the discovery of a new class of cardiovascular drug and the identification of their molecular target.

Overview of receptor-activated Ca^{2+} channel blockers

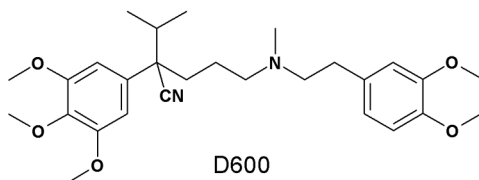
Receptor-activated Ca^{2+} influx channels (ROCCs) are not voltage-activated and have a broader cellular distribution than their voltage-operated counterparts.



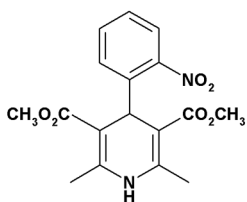
Verapamil



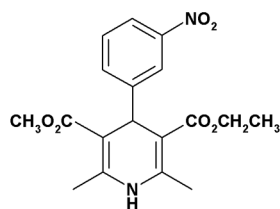
Prenylamine

Phenylalkylamines

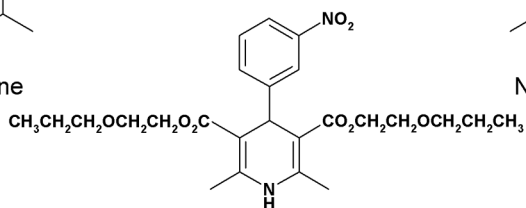
D600



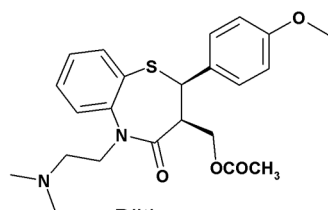
Nifedipine

Dihydropyridines

Nitrendipine



Niludipine



Diltiazem

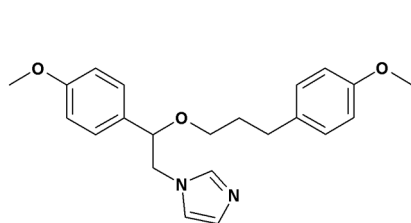
BenzothiazepinoneFIG 1. Structures of L-type voltage-operated Ca^{2+} channel blockers.

They are present in both electrically-excitabile cells such as muscle and nerve cells and electrically-unexcitable cells such as leukocytes and endothelial cells. Ca^{2+} entry through ROCCs has been most-intensively studied *in vitro* in leukocytes and leukocytic cell lines, both electrophysiologically and by using fluorescent Ca^{2+} indicators to monitor intracellular Ca^{2+} levels (Grynkiewicz et al 1985). These Ca^{2+} influx pathways include the highly Ca^{2+} selective current I_{CRAC} (Ca^{2+} -release activated Ca^{2+} current) in mast cells and lymphocytes (Hoth & Penner 1992, Zweifach & Lewis 1995) and Ca^{2+} -permeable non-selective cation channels in mast cells, (Franzius et al 1994), promyelocytic cell line HL60 (Krautwurst et al 1993), human neutrophils (von Tscharner et al 1986), monocytes and macrophages (Malayev & Nelson 1995, Naumov et al 1995).

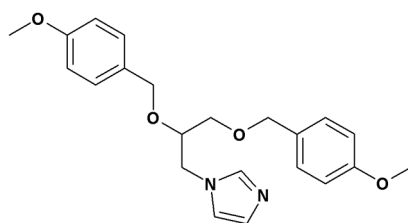
As expected, the realization of the functional importance of receptor-activated Ca^{2+} influx in leukocytes was not only of intense academic interest but was also of potential therapeutic interest in the development of anti-inflammatory drugs with novel mechanisms of action. After fluorescent intracellular Ca^{2+} indicators became available, several compounds were developed and receptor-activated/receptor-mediated/receptor-operated-store-operated Ca^{2+} channel/influx blockers—identified on the basis of their ability to inhibit Ca^{2+} influx in native cells using these Ca^{2+} indicators. These include SK&F 96365 and LOE 908 (Fig. 2). Their use as pharmacological tools has provided evidence of the heterogeneity of receptor-operated Ca^{2+} influx channels. However, unlike the situation with the L-VOCCs, none of these tools have been potent or selective enough to be used to isolate and identify the molecular target.

SK&F 96365, probably the best known synthetic ROCC blocker compound, inhibits receptor-activated Ca^{2+} influx and activation of platelets (Merritt et al 1990), neutrophils (Merritt et al 1990) and lymphocytes (Chung et al 1994). However, the molecular identity of the channels responsible for Ca^{2+} influx in the target cells was, and is still, unknown. The impact of not being able to identify this vital piece of information was to severely limit the ability of drug companies to develop therapeutically useful blockers of receptor-operated channels. SK&F 96365 was an optimized compound with improved potency over the prototypical compound SC38249 (Fig. 2) which was originally designed as a thromboxane synthetase inhibitor (Howson et al 1990). However the level of potency and selectivity vs. other channels e.g. L-VOCCs and Cl^- channels that could be achieved has not been sufficient to enable the progression from useful experimental pharmacological tool to therapeutic drug. The fundamental problem has been that, unlike the voltage-gated Ca^{2+} channel blockers, the molecular identity of the target channel was unknown and therefore no clear structure-activity relationship has been demonstrated.

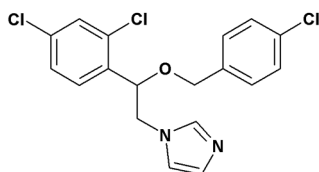
LOE 908, developed as an inhibitor of human neutrophilic ROCCs (Krautwurst et al 1993) has also proved to be a useful experimental tool. For



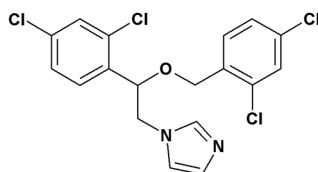
SK&F 96365



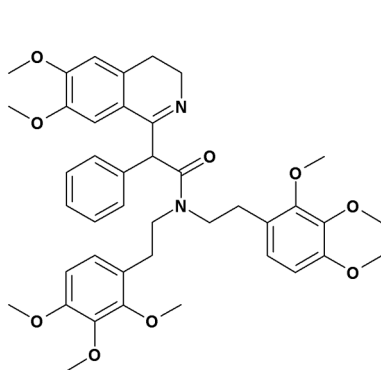
SC38249

Azoles

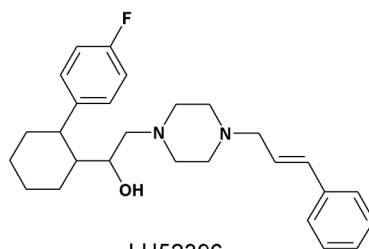
Econazole



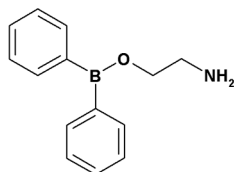
Miconazole



LOE 908



LU52396

Miscellaneous

2-Aminoethoxydiphenylborate (2-APB)

FIG. 2. Receptor-activated Ca^{2+} channel blockers; azoles and miscellaneous compounds.

instance, LOE 908 was used in combination with SK&F 96365 to demonstrate the presence of at least pharmacologically distinct Ca^{2+} -influx pathways in rat aortic smooth muscle cells (Iwamuro et al 1999).

A diverse range of compounds have also been found to inhibit receptor-operated Ca^{2+} influx. These include cytochrome P450 inhibitors, such as econazole and miconazole (Fig. 2) which are imidazoles like SK&F 96365 and which inhibit receptor-operated Ca^{2+} influx in a wide variety of cell types (e.g. neutrophils and platelets). There is evidence to suggest that the mechanism by which these compounds inhibit Ca^{2+} influx does not involve cytochrome P450 (reviewed by Clementi & Meldolesi 1996). LU52396 (Fig. 2), like SC38249, was designed as a thromboxane synthetase inhibitor and found to inhibit thromboxane-independent agonist-stimulated Ca^{2+} influx in platelets (Clementi et al 1995). More recently, 2-aminoethoxydiphenyl borate (2-APB) (Fig. 2) originally described as an IP_3 receptor antagonist has also been postulated to be a ROCC blocker (reviewed by Bootman et al 2002). However, as is the case with the more chemically complex organic channel blockers already mentioned above, 2-APB also shows little selectivity at the concentrations which block Ca^{2+} influx and therefore these effects can be difficult to attribute to its ROCC-blocking activity.

Inorganic divalent and trivalent cations are also widely used in the study of ROCCs. There are channel blockers e.g. Ni^{2+} , and the lanthanides La^{3+} and Gd^{3+} which can discriminate between ROCC Ca^{2+} -entry pathways (Itagaki et al 2002) and channel-permeant cations which are used as surrogates for Ca^{2+} influx as they are either not substrates for Ca^{2+} -efflux pathways (e.g. Ba^{2+} , Sr^{2+}) or interact differentially with fluorescent Ca^{2+} indicators (e.g. Mn^{2+}). The heterogeneity of native ROCCs is also supported by the differential permeability to Mn^{2+} (Demaurex et al 1992) and Sr^{2+} (Itagaki et al 2002).

In summary, there is much circumstantial evidence to suggest that each cell type can possess multiple Ca^{2+} influx pathways and that there is heterogeneity between cell types on the basis of the data obtained using the pharmacological tools available to date. To enable the full exploration and exploitation of the therapeutic potential of ROCCs requires identification of the molecular components of these channels and the development of more discriminating pharmacological tools with properties which allow their development as therapeutically useful agents.

Evolution of drug discovery approaches: implications for ROCCs

The completion of the sequencing of the human genome (Venter et al 2001) has provided molecular information about the candidate genes encoding these channel proteins and the escalation and miniaturization of drug discovery technologies in the last decade suggests that never before has there been a better chance for

identifying novel ROCC channel blockers. The combination of molecular information and increase in compound screening capabilities available now allows the use of a rational approach to systematically identify these elusive channels in the disease-relevant cells to identify modulators of these channels which could fully exploit their therapeutic potential.

TRP channels: targets with real potential for drug discovery?

The discovery of human homologues of transient receptor potential (TRP) channels in the middle of the last decade has provided to date the most promising molecular candidates for these elusive ROCCs. Within this gene family there are now three main subfamilies encoding approximately 20 cation channels. Most are non-selective cation channels, whilst there are a few which show selective permeability for certain divalent cations, e.g. Ca^{2+} or Mg^{2+} (Clapham et al 2001, Montell et al 2002).

The canonical TRP channels, the TRPC subfamily, are the original members which were discovered and are homologous to the *Drosophila trp* channel—the founder member of the TRP gene family and shown as a receptor-activated Ca^{2+} -permeable channel. Members of the TRPC family still represent some of the best candidates for ROCCs by virtue of their close similarity of electrophysiological fingerprint, pharmacological sensitivity to certain native ROCCs e.g. the $\alpha 1$ -adrenoceptor-activated non-selective cation channel ($\alpha 1\text{AR-NSCC}$) in portal vein smooth muscle (Inoue et al 2001) and the functional importance as gene-specific knockdown of TRPC6 expression reduces $\alpha 1\text{AR}$ -mediated NSCC current and Ca^{2+} influx. A role of TRPC6 in vascular smooth muscle function has also been suggested in rat cerebral artery myogenic tone (Welsh et al 2002) and also in pulmonary artery smooth muscle proliferation which is a component of pulmonary hypertension (Yu et al 2003).

TRPC6 expression is particularly high in mouse and human lung (Boulay et al 1997, Riccio et al 2002). More detailed analysis of specific tissues of the lung have indicated the presence of TRPC6 mRNA and protein expression in human airway smooth muscle (Corteling et al 2003) suggesting that in addition to having a role in vascular smooth muscle function, TRPC6 may also be involved in bronchial smooth muscle contraction. Therefore TRPC6 channel blockers may represent a novel mechanism for the development of bronchodilator therapy. Gorenne et al (1998) demonstrated that ROCC-mediated Ca^{2+} influx contributes a major source of Ca^{2+} required for bronchoconstriction of human bronchioles stimulated by a wide range of spasmogenic agents, e.g. LTD_4 , allergen, acetylcholine and histamine. The epithelial cells of the lung lining the airway lumen and submucosal glands both strongly express TRPC6-immunoreactive staining (Corteling et al 2003). As epithelial cell Cl^- ion secretion can involve increases in

cytosolic Ca^{2+} levels (Chiyotani et al 1994, Ko et al 1997) and receptor-stimulated Ca^{2+} influx has been demonstrated in well-differentiated human bronchial epithelial cells (Meshner et al 2003), TRPC6 may also be implicated in epithelial cell function.

Leukocytes are one of the cell types in which ROCCs have been most intensely studied and ROCC-mediated Ca^{2+} most widely documented, and it is not surprising that many TRP channels have been shown to be expressed in these cells (reviewed by Li et al 2002). Because many leukocyte responses such as release of inflammatory mediators and degranulation which cause tissue damage have a ROCC-mediated Ca^{2+} -influx-dependent component, modulation of Ca^{2+} influx in these leukocytes is a potential anti-inflammatory approach. With respect to inflammatory diseases such as chronic obstructive pulmonary disease (COPD) and asthma, the main orchestrative leukocytes involved are the lung macrophage (Barnes 1998) and the CD4^+ lymphocyte, respectively (Hamid et al 1991), whilst the main infiltrating granulocytes are neutrophils in COPD and eosinophils in asthma. Amongst the TRPC members, TRPC6 is the mRNA and protein for which there are clearest data to suggest its presence in a range of leukocytes such as the lymphocyte (Gamberucci et al 2002, S. Li, unpublished data), the neutrophil (Heiner et al 2003, S. Li, unpublished data) and the lung macrophage (S. Li, unpublished data). However, it remains to be determined what the functional importance of TRPC6 is in these leukocytes.

Other TRP channels which may also be of relevance to COPD and asthma include TRPM2 which appears to be selectively highly expressed in granulocytic and monocytic cells (Sano et al 2001, Heiner et al 2003). It is activated by hydrogen peroxide and has been suggested to play a role in oxidant stress (Wehage et al 2002). The presence of TRPV4 protein has also been demonstrated in lung macrophages, epithelium and in tissue neutrophils (Delany et al 2001).

Concluding remarks

Antagonists of L-VOCCs in electrically-excitable cells are valuable therapeutic agents for the treatment of cardiovascular diseases. It is also clear that Ca^{2+} -permeable channels are functionally important in electrically non-excitable cells such as leukocytes and potential targets for therapeutic intervention. Previously, progress in this area has been hindered by the lack of information about the molecular identity of these targets. However, members of the recently discovered TRP gene family represent promising molecular candidates for these channels. The challenge now is to identify which TRPs are functionally important in disease-relevant cell types and to fully explore and exploit them as drug targets. Due to the molecular information and tools now available, we are in a better position to meet this challenge.

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DISCUSSION

Ambudkar: I liked those experiments in which you were looking at polarity to see which direction the Ca^{2+} was coming in. It was nice to see basolateral Ca^{2+}

influx: this is quite consistent with previous studies in polarized epithelial cells. You found TRPC6 in the apical membrane, however. Would you therefore conclude that the UTP-mediated Ca^{2+} influx is not coming through TRPC6?

Li: I don't think we can conclude that without knocking out TRPC6. It could be due to the sensitivity with which the antibody can detect low levels of TRPC6 located intracellularly within the epithelium. Although TRPC6 looks to be mainly apical, there is some staining deeper down in the epithelial layer. But how many functional TRP channels are needed in order to see a functional effect?

Ambudkar: Have you looked at other TRPCs? Are there any in the basolateral membrane? We find TRPC1 in basolateral membrane in submandibular gland acinar cells, which are also nicely polarized.

Li: We found TRPC1 message, and this was actually higher in the differentiated human bronchial epithelial cells (HBECs). But we haven't stained with anti-TRPC1 antibody to see where this is localized.

Muallem: This experiment is pretty controversial. It has now been done by two different prominent groups, and they get completely opposite results (Paradiso et al 1995, Gordjani et al 1997). Paradiso et al (1995) got influx from both membranes and Gordjani et al (1997) have similar results to yours. The story might depend on culture conditions and so on.

Li: There could be regional differences depending on the location within the lung from which the epithelium was removed. It could also be agonist dependent: we have only used one stimulus. It might be interesting to use other stimuli such as methacholine.

Putney: The conclusion in the Boucher paper (Paradiso et al 1995) was that the agonist activation showed sidedness, but thapsigargin activation did not. Assuming that these are store-operated channels (SOCs), the suggestion is that there could be spatial restriction on where IP_3 would act. Up until that point, a lot of the work on diffusion constants for IP_3 had always suggested that IP_3 should act globally.

Muallem: With the work from Gordjani et al (1997), no matter how they tried to deplete the stores they could never get Ca^{2+} influx across the apical membrane.

Putney: Have you tried using thapsigargin on this preparation?

Li: Yes, but we had a practical problem of it sticking to everything. We will try doing the experiment with cyclopiazonic acid (CPA).

Nilius: I found it intriguing that in your polarized cells TRPV4 is by far the most prominent channel. It fits marvellously to our data: EETs are involved, you get a signalling pathway with bradykinin. These data are all converging on TRPV4 activation. So why do you still look for TRPC6?

Li: We started with TRPC6, but we are moving on to look at other TRPs including TRPV4.

Nilius: From your data, TRPV4 is a marvellous target.

Li: I think it looks very interesting. I would like to see whether TRPV4 protein is expressed in these differentiated HBECs too. At the moment I don't know where it is located, and I would be interested to see whether it has differentially localized apically and basolaterally.

Scharenberg: In your quantitative PCR surveys did you see TRPM2 expression in any subsets of lymphocytes, besides mouse cells?

Li: We have only just started looking at TRPM2, so we haven't done profiling in a huge range of cell types. We haven't looked at CD4⁺ and CD8⁺ T cells derived from human blood yet, but I think our preliminary data on a sample of mixed lymphocytes suggest that TRPM2 is expressed at quite low levels.

Authi: Do the TRPC6 variants you have detected have any relevance? Certainly the one you pointed out is lacking a transmembrane domain.

Li: I don't know what the functional relevance is of these splice variants.

Authi: Have you looked for these in any disease states?

Li: No.

Putney: Was your conclusion that the SK&F compound doesn't distinguish between the store-operated and receptor-operated channels?

Li: I wasn't trying to differentiate between the two types of channels.

Putney: But LOE does to a large extent?

Poll: That influx pathway in human bronchial epithelial cells is relatively insensitive to both econazole and SK&F 96365.

Li: We were going to look at LOE 908 but we have been using fura-2, and LOE 908 interferes with fura-2 measurements so we would have to change the fluorophore. This is something we are planning to do.

Putney: One observation we made is that in HEK cells when we compare the store operated entry and TRP3, the SK&F compound has some selectivity for TRP3, with a difference of around 1–1.5 orders of magnitude. In the same way that we can use gadolinium to selectively remove the store operated entry and leave the TRPC3, we can do the opposite experiment with SK&F. I think Colin Taylor has some evidence that for real receptor-operated channels, whatever the signal is, it also has that selectivity.

Taylor: It is a very similar story in that low gadolinium and 2-APB will take out capacitative entry and not touch the receptor-activated entry. LOE and SK&F will do the opposite.

Authi: There was a compound recently described called TRIM, which is reported to block SOC activity in anococcygeus smooth muscle (Gibson et al 2001). Have you tried this?

Li: No.

Authi: It was originally described as an inhibitor of NOS activity. This compound has no effect on voltage-gated channels.

Muallem: I have a question for the drug company people. How are you going about drug development with all these completely non-selective drugs?

Li: We have to prioritize and start with the channels that we are most interested in first and target those.

Poll: Presently, TRP pharmacology is sparse and the tools are rather blunt, as we have been discussing. However, running 20 or so TRP high throughput screens would not be easily possible! One approach is to obtain functional data in cell types of interest, for example, by using a gene knockdown approach such as siRNA. This in addition to the evidence we have for cellular and tissue distribution and molecular epidemiology should help us to select a rather more limited panel of TRP channels to run high-throughput screens against to try to get more potent selective tools.

Putney: There are two strategies. You can start with physiological phenomenology and screen libraries, or you can start with a molecule and screen in that direction. I don't know which is best. To me, there is a lesson from the dihydropyridines: the original guess was that these couldn't possibly be useful because Ca^{2+} channels are everywhere. The physiology came later, and they established the subtypes of channels and the specificities. I wish you guys would take your libraries and throw them at the SOCs and ROCs and see what happens.

Penner: I admit that I am amazed that you guys are targeting TRPCs. Don't you do target validations?

Westwick: It all depends on what you mean by 'target validation'. This doesn't just apply to TRPs. You can take the reverse approach: look at the story of how the dihydropyridines came about. They were used later to validate that certain VOCs were involved in certain cells, and not the other way round. We can do it by trying to set up screens where we believe what we are measuring is due to the channels we put in as opposed to those that were already there, or we can just work our way through with siRNA, depending on what function you want to look at. You demonstrate at the same time that you are specifically reducing the protein, the current and what we are more interested in, the downstream functional effect. It is still a pretty long-winded approach.

Penner: Pharmaceutical companies have usually taken the 'FLIPR' (Fluorometric Imaging Plate Reader) approach. They screen for a Ca^{2+} -dependent process, and come up with a compound. Then they work out which target the compound acts on.

Westwick: That is what is referred to as the 'black box' system. You have a readout, you find a compound, but you have no idea of what it is hitting. It takes you a long time to find what that compound hits, which you need to do if you want to optimize it.

Gill: That is one approach.

Westwick: It is, but it is terribly difficult to identify the target once you find some functional effect of a compound.

Gill: As long as you have some function that can be measured, and you have a large enough throughput, you can screen enormous numbers of compounds.

Li: The problem is, how can you actually optimize the compounds?

Westwick: Both approaches are being taken, but I would favour working with a system where you know that a particular TRP or combination of TRPs is actually responsible for your output. If you want to set up a binding assay with the GPCRs you don't need an intact system, but for anything with ion channels you will need an intact system, which by the nature of it is very complex and your compound can hit a range of sites and molecules.

Barritt: Could I try to clarify in my mind your arguments. Are you saying that if, for example, you could transfect cell lines stably with each TRP protein and you could search your library of potential inhibitors, you might find some interesting compounds? However, you would not know what the possible physiological consequences were? Are you saying that you wouldn't do these screens on singly-expressed TRP channels because you might come up with an inhibitor, but you don't know what its physiological action is? I think you are saying that you need to know what the physiological actions of a given TRP channel are before you go to look for inhibitors.

Westwick: Yes. The other problem with the TRPs concerns some of the issues we have been discussing today. We have no direct activators of them. That's a difficulty. If we go back to the vanilloid receptor, the VR1s were screened just like you say and compounds were produced as a result of that.

Li: But then there was an actual ligand.

Penner: They are all capsaicin antagonists; they are not VR1 antagonists.

Westwick: That was based on capsaicin binding. There are compounds which block VR1 which are not capsaicin inhibitors.

Poll: As long as you do some level of molecular validation, that is OK. You don't need to fundamentally understand the physiological role of molecular targets before you do a high throughput screen. But we need to know target cell types of interest. This is the sort of level of evidence to stimulate us to run a high throughput screen to get the tools that will give us further clues as to the function. It is a balance between these two approaches.

Kunze: What I suspect you are saying is that you are going to target a disease or problem that you think is good for the drug company to pursue. For instance, the VR1 is an important one with its role in pain. So, are you going to look for a function and then see how the TRPs fit into that function?

Westwick: You can do it either way. In the beginning, whether it is a TRP, a kinase, a GPCR or a phosphatase, you want to see whether there is a change between the disease and normal states. I am still not convinced this is the case

with the TRPs, particularly if we are interested in inflammation. There are very few data in terms of any change in expression of TRPs. This is why we are interested in the differentiated epithelial cells. If we can back this up with the antibodies we are generating to these and show differences in protein and electrophysiology between the undifferentiated and differentiated cells, this is something that is worth targeting.

Putney: A particular molecule doesn't have to be part of the disease to be a part of the cure.

Westwick: But it would enhance the selectivity of your compound if it is only expressed in disease.

Putney: In many ways it seems like this is the toughest thing to do. If you have a molecule that is mutated or under regulated, it is very hard to reverse that. On the other hand, it seems it would be much easier to choose another pathway.

Westwick: Again, I am not arguing that that is the only way. You can just demonstrate that you think it is physiologically relevant, you develop an assay and find a compound. You can find a compound that works in the assay but then to optimize that compound that you want to give orally, you really have to understand your assay and the target which it is working against. The curious thing is, if you go back to the dihydropyridines there was very little known about what was operating Ca^{2+} channels when Bayer found nifedipine. They actually used the dihydropyridines to characterize the existence of the different VOCCs.

Putney: It is also an example of where the molecule we treat is not the cause of the disease.

Westwick: But it is only by good fortune that those particular dihydropyridine-sensitive VOCCs are only in those tissues that have an effect on hypertension. Otherwise we would have had major gut and airway effects, for example.

Poll: That is an interesting point. If we were to discover L-type VOCCs now, knowing their distribution we might predict that there would be rather serious consequences of using a VOCC blocker. This sort of information could inhibit us. The fact that VOCC blockers were discovered to be Ca^{2+} antagonists by looking at functional cardiovascular changes led us subsequently to realise that you could have some selectivity with such an agent.

Putney: I won't beat this to death. I was pushing this idea of trying to get SOC blockers years ago with companies, and the reaction I got is that they are everywhere so this would be a disastrous approach. We now know that they are certainly not the same everywhere, and when everything is worked out at the molecular level, everyone can jump in at that point.

Benham: The thing that the folks who aren't used to working with chemists optimizing compounds probably don't appreciate is how robust the assays must be before chemists are happy with them. You don't make improvements in

compounds in great leaps. Everything tends to be done in minute increments. It is a question of optimizing the compound at half a dozen or more different sites. To be able to do this you need an assay sufficiently robust to do large numbers of compounds and that is reproducible. We get into trouble if our assays go out by less than half a log unit.

Penner: If you use a heterologous expression system you can optimize it.

Benham: I did this 15 years ago with Tim Rink and Trevor Hallam, and ended up producing SK&F 96365. This was about as far as we got. We got that far relatively quickly, and then we hit a brick wall because we were dealing with a black box. We had no idea what the molecular basis of the channels was. We didn't know whether the effects we saw in neutrophils were due to the action upon the same proteins as in the platelets, and we didn't understand the difference between store-operated and receptor-mediated Ca^{2+} influx. So we ended up with very confusing structure activity relationships because we were probably looking at multiple molecular pathways contributing to our Ca signals. In retrospect, it's not surprising that SKF is non-selective as non-specific compounds are the most effective blockers in these complex systems.

Cox: If you had done the black box approach now you would have come up with the same molecule, and you wouldn't be able to take it anywhere because it hits everything at a low concentration. A chemist isn't going to be able to optimize it any further. Where do you go with it?

Benham: Another problem that is relevant to channels generally is that we are competing for resources against folks who have enzymes as targets that they can crystallize and get good structures for both the bare enzyme and the enzyme with their lead molecules bound. This enables them quickly to generate molecular models of the pharmacophore. Coupled with rapid high throughput screening this means that they can make phenomenal progress in getting from micromolar to picomolar affinities.

Cox: With the SK&F compound, now you have perhaps an insight into the various channels you could go back and think of that compound and all the analogues to see whether you can tease out any structure-activity relationships, albeit very small ones. This wouldn't have been possible before because you didn't have the separate channels to play with.

Penner: SK&F is so non-specific. It is not even specific among the TRPs. It will even block Cl^- channels.

Cox: Presumably there is a pool of analogues. You could look for a fingerprint for each molecule and try to build up a direction to send you down from that particular lead i.e. from a diverse set of analogues, differences in activity across a range of the molecular targets can be exploited to make new compounds with further differential activity (selectivity) or even specificity for a given single target.

Benham: If we got SK&F as one of our hits today, we would probably discard it as a way forward. You would hope to get much better hits than this now when there are a million compounds running through a screen with a lot more diversity in the molecules.

Montell: If you are committed to doing screens, a potentially useful approach is to look for effects resulting from overexpression of random genes obtained from expressed sequence tag (EST) libraries. This would be analogous to screens that are done in flies using the GAL4/UAS system.

Westwick: This has been used in zebrafish as well.

Gill: What about the knockouts? Is anyone making a TRPC3 knockout?

Westwick: Don't we need conditional knockouts?

Putney: People either have already, or are trying to make knockout mice for all the TRPCs, and I assume this also applies to TRPVs and TRPMs.

Muallem: Cre would be a better way to go.

Scharenberg: Developing Cre promoter mice takes time and money. It is non-trivial.

Westwick: From the work we have seen today about the up-regulation of TRPC3 in the TRPC6 knockout, was that due to a developmental change? If you did siRNA in a particular cell type would you also see the same result? Would you get more TRPC3 when you lose TRPC6? There are a lot of people doing genome-wide siRNA: they are taking all 40 000 or so genes, targeting with siRNA and then looking for a functional output. They work back to find out which enzymes or channels the genes are directed against. But it is clear from what we have heard here that this is a risky business. Even if you might knock one down, you might up-regulate half a dozen at the same time.

Scharenberg: It is more a problem in mouse development. There are more opportunities for changes than there are in culture. Culture has its own problems. Choose your poison.

Putney: Whenever you make a mutant mouse you are very fortunate if it doesn't turn you into a developmental biologist!

Gudermann: Every approach has its own advantages and disadvantages. The way we try to find the solution is that we are now constructing transgenic mice that inducibly express dominant negative channel subunits whose expression can hopefully be switched on in the animals fast enough. But if you talk to people who work on PKC they tell us that even within a couple of hours you see down-regulation of one and up-regulation of the other isoform.

Putney: The strong conclusions come about when you try many different approaches and they all lead to the same answer.

Scharenberg: The question is, is it easier to make the mice, or to do the screen, make the drug and then come back and check?

Gill: I don't think it is either or.

Hardie: In *Drosophila* we also have temperature-sensitive alleles which give a reversible knockout in a timescale of seconds to minutes. This is a very nice tool.

Zhu: Say, for example, that we want to know whether there is a drug that specifically targets TRPC3. Is this something that should be screened for now, or should we wait until we find the specific composition of the channel?

Putney: That is a good example. You could set up a screen tomorrow for TRPC3, 6 and 7. It would be pretty robust. But the concern here is that if we got the world's very best TRPC3 blocker, it wouldn't be good for anything if we couldn't optimize it.

Westwick: There are likely a number of companies that have set up screens like this and do have compounds. All I was indicating was caution in terms of what happens with them.

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