Small bone-building fragments of parathyroid hormone: new therapeutic agents for osteoporosis

James F. Whitfield and Paul Morley

The brittle, fracture-prone bones of an osteoporotic postmenopausal woman are the products of an excessive uncompensated resorption of trabecular bone by osteoclasts. Osteoporosis is currently treated with the osteoclast suppressors calcitonin, bisphosphonates, or oestrogen, which stop further bone resorption without stimulating new bone growth. Here, **James Whitfield and Paul Morley** review the growing evidence that small adenylate cyclase-stimulating fragments of the parathyroid hormone are promising therapeutic agents for osteoporosis that potently stimulate osteoblasts to make mechanically strong or supranormally strong bone.

The mature human skeleton is being continuously remodelled to repair microfractures resulting from normal use and to maintain optimal load-bearing configurations. The skeleton is turned over completely every eight to ten years by the operations at any given moment throughout the skeleton of 3×10^7 teams of bone-resorbing osteoclasts and bone-building osteoblasts known as basic multicellular units (BMUs)[1–4]. Each 50- to 100-day BMU cycle is stochastically triggered by some unknown signal(s), which recruits a team of osteoclasts that moves along a small patch of bone at a rate of 25 μm day^{-1}, digs a 230–430 μm tunnel in the bone's compact cortical envelope or a trench in the surface of a strut or plate of the bone's internal trabecular lattice, and liberates factors such as insulin-like growth factor I (IGF-I) and IGF-binding protein 5 (IGFBP-5). These factors had been deposited in the matrix by the osteoblasts of a previous BMU and now recruit a succession of immobile groups of osteoblasts that try to refill the excavation with new, factor-loaded bone (Fig. 1a)[1–4]. However, the osteoblasts never quite refill such excavations, and the resulting remodelling deficits cause a steady loss of bone in the years following the peaking of the bone mass that occurs in early adulthood[1–5]. If a woman does not make enough bone during her youth to keep her well above the fracturing threshold, the mounting remodelling-produced bone loss will make her susceptible to a crippling consequence of menopause, namely osteoporosis or brittle bone disease.

When a woman's oestrogen production markedly falls at menopause, the BMU activation frequency doubles and the osteoclasts start digging deeper, but the osteoblasts can't cope with this and the remodelling deficits escalate, most importantly for the woman's mobility, in the trabecular lattices of her hip and spine (Fig. 1b)[1–6]. The osteoclasts now weaken these bones by disconnecting and destroying, for example, the horizontal struts that interconnect and prevent excessive bending and breakage of the thicker, load-bearing, vertical columns of the trabecular lattice (Figs 1b, 2)[1–3]. Eventually, a bone 'mechanostat' mechanism detects the increasing strain resulting from the disruption and weakening of the trabecular lattices and lowers the BMU activation frequency[2], but it is usually too late and the woman is left with a fracture-prone hip and spine, and is still losing bone, albeit more slowly[5,6].

The increased osteoclast activity, and the decline past the fracturing threshold into osteoporosis, can be slowed down, or arrested, by suppressing osteoclasts with agents such as nasally delivered calcitonin, an oral bisphosphonate, or oestradiol-17β, whose disappearance triggered the increase in activity[5,6]. However, these agents do not stimulate osteoblasts to produce strong new bone, they only prevent further loss[2,5,6]. What is required is a drug that safely, selectively, strongly and directly stimulates trabecular growth, and thus strengthens the remaining trabecular lattices in the fracture-prone bones of an osteoporotic patient when given nasally, orally, or at least subcutaneously (like insulin) in small doses. Such an 'anabolic' drug, which is now attracting considerable interest in the pharmaceutical industry, was discovered 63 years ago; it is parathyroid hormone (PTH).

PTH, an osteogenic paradox

According to conventional wisdom, small pulses of PTH (one to five per hour) maintain the blood Ca^{2+} concentration at its normal value by maintaining Ca^{2+} recovery from the glomerular filtrate of kidney tubule cells and the activity of renal 1a-hydroxylase, which produces 1a,25$(OH)_2$ vitamin D_3, and this promotes Ca^{2+} uptake by intestinal epithelial cells[7,8]. However, hypersecretion of PTH or injection of pharmacological doses of the hormone stimulates the recruitment of osteoclasts, which destroy bone and place its Ca^{2+} into the blood. It would be unusual for a resorption stimulator like this to be used to stimulate bone growth. However, Hans Selye published a paper in 1932 in which it was showed that PTH could and, moreover, very effectively did have such an effect[9]. It was found that intraperitoneal and intermittent (once every second day for one week) injection of a small dose of a crude bovine parathyroid extract dramatically increased osteoblast numbers and trabecular bone formation in the femurs of normal 14–30-day-old albino rats. In fact, this intermittent treatment was so osteogenically potent that it almost produced osteopetrosis, or 'marble-bone', the bone marrow-strangling antithesis of osteoporosis[9]. Since this bone growth was triggered by injecting a parathyroid extract, it could have been the result of either the extract's PTH or a potent osteogenic contaminant.

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An increasing number of reports (Fig. 2) have unanimously confirmed the 'Selye effect', mainly in ovariectomized rats, but also in dogs and humans, mostly using a fully bioactive synthetic PTH fragment, human PTH(1–34) [hPTH(1–34)][10–38]. The sexually mature, ovariectomized rat is a widely used model of human postmenopausal osteoporosis that is now required by the United States Food and Drug Administration for preclinical evaluation of agents for treating postmenopausal osteoporosis[39]. For example, ovariectomy of a Sprague-Dawley rat triggers a 75% decrease in the total mass and Ca^{2+} content of the femoral trabecular (but not cortical) bone between the second and ninth week after the operation[10,29,35,36]. So far, all agents that stop resorption or stimulate trabecular bone growth in the ovariectomized rat model do the same in humans[10,16].

Thus, it is now certain that single daily subcutaneous injections of a small amount (for example, 8–25 nmoles kg^{-1} of body weight) of hPTH(1–84) holoprotein or its hPTH(1–34) fragment, which are too small to affect the blood Ca^{2+} concentration, can strongly stimulate the production of mechanically normal or even supranormally strong cortical and trabecular bone in the femurs, tibias and vertebrae of immobilized or ovariectomized rats (Figs 1c, 3)[10–14,19–25,27,29–38]. The high biomechanical quality of the new bone is indicated, for example, by the supranormal compression tolerance of femurs and vertebrae from hPTH(1–34)-treated ovariectomized rats when the bones are appropriately prepared and compressed[11,22,24]. However, the fragment cannot create new trabeculae to replace lost ones. It can only increase the thickness and reinforce the connectivity of the remaining trabeculae. Most importantly, one subcutaneous injection of 50–100 μg of hPTH(1–34) kg^{-1} of body weight every day for 6–24 months into osteoporotic men and postmenopausal women significantly increases the vertebral trabecular volume and mineral density by 32 to 98% either without affecting, or only slightly reducing (for example, 5–7%), cortical bone density[10,14–18,21,26,28,33].

The occurrence of some PTH-induced cortical bone loss in some, but not all, human osteoporotic patients, as opposed to the cortical growth in PTH-treated ovariectomized rats, has dampened enthusiasm for PTH therapy[10], but this concern is unwarranted as it now appears that any initial cortical bone loss will 'spontaneously' disappear during prolonged PTH treatment[14]. Nevertheless, it must be said that inability of PTH to stimulate cortical bone growth in humans would limit its effectiveness, because femoral-neck fracture, unlike vertebral-crush fracture, is due mainly to cortical rather than trabecular weakening and hPTH(1–34) treatment does not seem to increase hip-bone density[6,16,40]. This difference between the response of rat and human cortical bone to PTH treatment is not understood, but it may somehow be related to the fact that rats do not have Haversian bone[10]. However, the combination of an anabolic PTH fragment and a resorption blocker may ultimately prove to be the most effective treatment for osteoporosis.

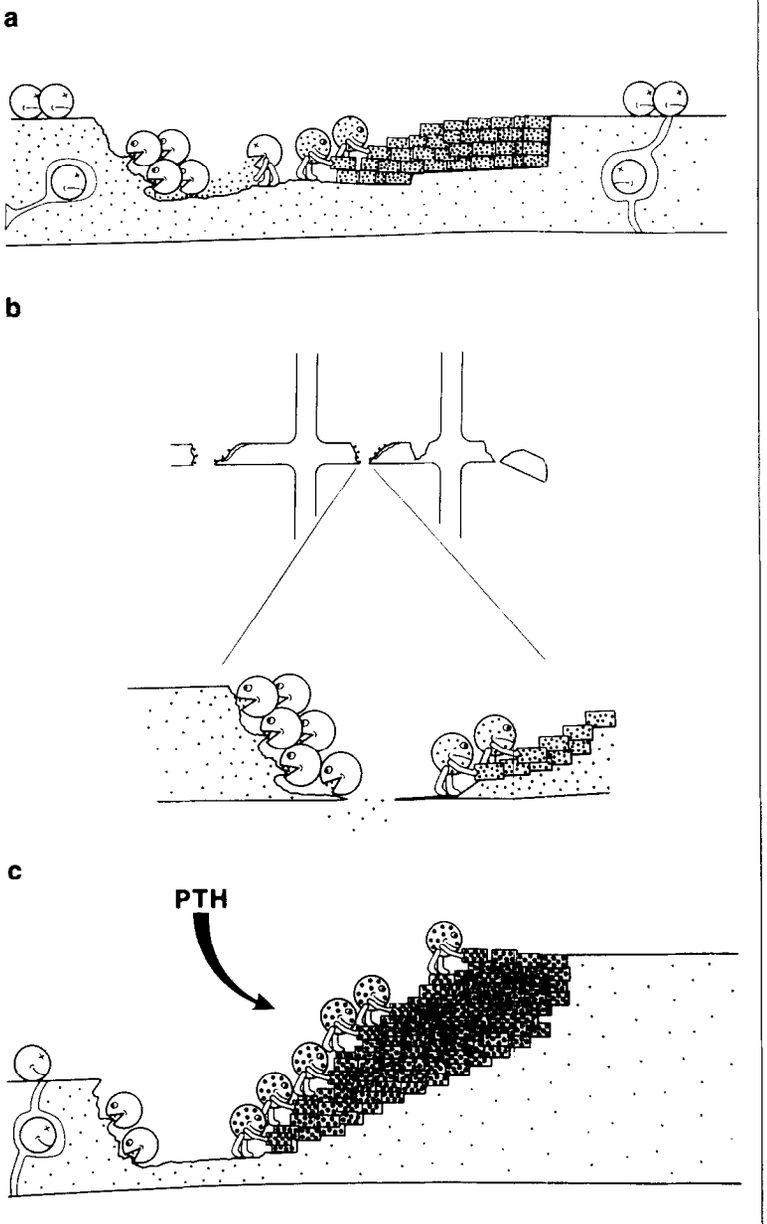

Fig. 1. Bone remodelling and osteoporosis. **a:** A normal basic multicellular unit consisting of mobile osteoclasts resorbing bone and releasing factors (dots) from the bone matrix as they move along the bone. The factors recruit and stimulate successive teams of osteoblasts that, anchored in one place, try to refill the cavity with new bone loaded with factors to activate a future basic multicellular unit. **b:** A menopause-triggered increase in osteoclast activity weakening a vertebra by perforating one of the horizontal struts of the bone's internal trabecular lattice. **c:** Parathyroid hormone (PTH) triggers bone growth through a cAMP-mediated production and secretion of autocrine and paracrine factors, such as insulin-like growth factor I and insulin-like growth factor-binding protein 5 (dots) that stimulate osteoblast precursor proliferation and production of bone constituents by mature osteoblasts.

Osteogenic trigger for PTH

The question arises as to how PTH triggers bone growth. The answer to this question could be the key to making the smallest, cheapest, and functionally simplest second-generation PTH fragment or analogue for reversing the bone fragility in osteoporosis with the least number of side-effects. PTH could operate by activating the adenylate cyclase–cAMP-dependent protein kinases mechanism, the phospholipase Cβ (PLCβ)–Ca^{2+}–protein

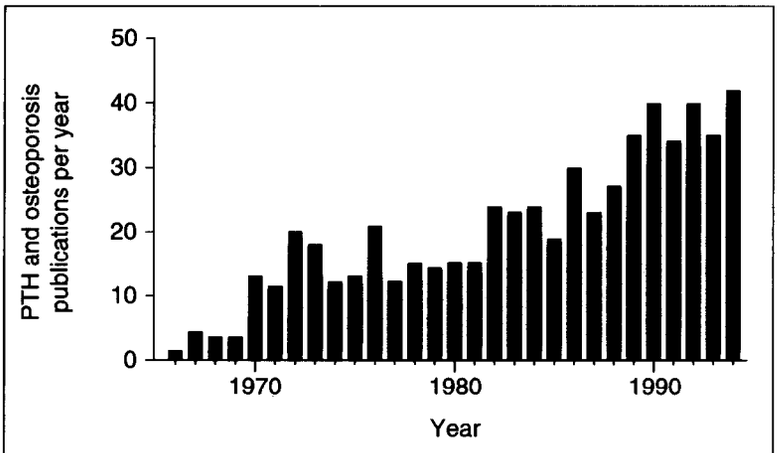

Fig. 2. The increasing interest in parathyroid hormone (PTH) in the treatment of osteoporosis.

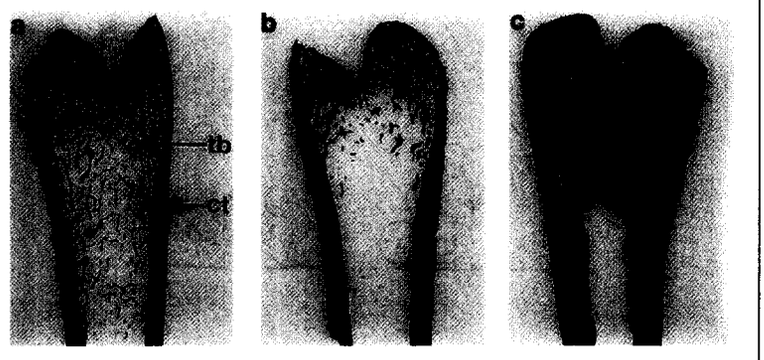

Fig. 3. An example of the ability of human parathyroid hormone (1–31)NH_2 [hPTH(1–31)NH_2] to stimulate trabecular bone growth in an ovariectomized rat. **a:** A demineralized distal femur from a normal three-month-old rat; **b:** distal femur with its depleted trabecular bone at eight weeks after ovariectomy; **c:** distal femur with dramatically thickened trabecular bone at eight weeks after ovariectomy and 42 daily injections of 10 nmoles of hPTH(1–31)NH_2 kg^{-1} of body weight from two to eight weeks. The 10 μm sections of demineralized bone were stained with Sanderson's rapid bone stain. ct, cortical bone; tb, trabecular bone.

kinase C isoforms (PKC) mechanism, or both, in osteoblasts or osteoblast precursors; this is because its G protein-coupled receptor couples independently to the adenylate cyclase-activating G_s protein and a PLCβ-activating G_q protein (Fig. 4)[41]. It has long been known that PTH needs the first two N-terminal amino acids and some part of the amino acid 25–34 region to activate the adenylate cyclase-stimulating G_s protein, and more recently it has been demonstrated that only the 29–32 region is required to activate G_q and stimulate PLCβ and PKC (Fig. 4)[42,43]. Thus, both hPTH(1–84) and hPTH(1–34) stimulate two primary signalling enzymes in osteoblasts. A set of single- and dual-signalling fragments (Fig. 4) were tested for their abilities to stimulate bone growth in the ovariectomized rat model. One of these was hPTH(1–31)NH_2, the first PTH fragment to stimulate adenylate cyclase as effectively as hPTH(1–84) and hPTH(1–34), without being able to stimulate the PLCβ and PKC mechanism because of its truncated 29–32 region[30,42–44].

At the outset it was expected that it was the PLCβ- and PKC-activating amino acids 29–32 region of the PTH molecule, and not the adenylate cyclase-activating two N-terminal residues, that would be needed to trigger osteogenesis, because Somjen and co-workers had shown that PTH needed the 28–34 region, but not the first two N-terminal amino acids, to stimulate DNA replication by rat osteoblasts *in vivo* and *in vitro*[45]. However, the results were as surprising as they were unequivocal when the fragments were tested on 3- and 12-month-old ovariectomized rats. One subcutaneous injection of the adenylate cyclase-stimulating hPTH(1–84), hPTH(1–34) or the new hPTH(1–31)NH_2 at a dose of 8 nmoles kg^{-1} of body weight (each day for six weeks starting two or nine weeks after ovariectomy) caused a two- to fourfold increase in the trabecular Ca^{2+} content, total trabecular bone mass and the mean trabecular thickness in the distal femur[30,35,36]. Starting the daily injections with a larger dose (10 nmoles kg^{-1} of body weight) of hPTH(1–34) or hPTH(1–31)NH_2 two weeks after ovariectomy, when the trabecular bone was still relatively normal, caused a massive trabecular build-up with a corresponding shrinkage of the lattice's marrow spaces[30,35,36] (Fig. 3c), just as described by Selye[9]. In contrast, isomolar doses of fragments such as 1-desamino-PTH(1–34) and hPTH(8–84), which stimulate PLCβ and PKC, but not adenylate cyclase, had no effect[30]. This has now been confirmed by Strein, who found that hPTH(28–48), which stimulates PLCβ and PKC but not adenylate cyclase[42,43], did not stimulate tibial growth in ovariectomized rats, while hPTH(1–34) was significantly osteogenic in the positive control ovariectomized rats[33].

These results have clearly established that daily PTH-triggered pulses of cAMP-dependent protein kinases activity are the sole primary triggers of PTH's potent anabolic action in bone. But how might they do it? The available evidence is consistent with PTH stimulating a subpopulation of bone cells bearing PTH receptors to express the cAMP-responsive genes coding for autocrine and paracrine anabolic factors, such as IGF-I and IGFBP-5 which, in turn, stimulate the proliferation of osteoblast precursors and the differentiation of the growing pool of precursors into active bone-forming osteoblasts (Fig. 1c)[30,35,36,46,47]. However, while intermittently injected small doses of PTH stimulate bone cells to produce a set of autocrine and paracrine osteogenic factors, stronger sustained PTH stimulation causes osteoblasts to express a different set of factors such as collagenase, granulocyte–macrophage colony-stimulating factor, interleukin 6, plasminogen activator (to activate latent collagenase), and PGE_2, which collaborate to recruit and activate bone-resorbing osteoclasts (for review see Refs 30, 35, 36).

Future directions

Although hPTH(1–84) can now be mass-produced by recombinant technology and is in clinical trials for osteoporosis treatment, it will be replaced sooner or later by a much smaller 'designer' fragment for stopping bone loss and strengthening disrupted trabecular lattices in osteoporotic postmenopausal women. It is fortunate for efforts to minimize cost and the needed amount of injectable

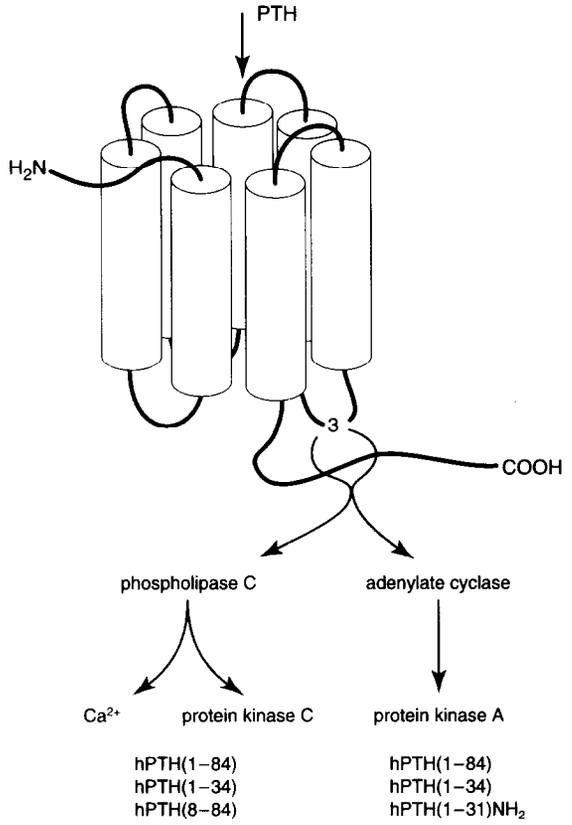

Fig. 4. The receptor activated by parathyroid hormone (PTH) can independently activate one or two signal mechanisms depending on whether the ligand has an intact N-terminus or the 29–32 amino acid region. Compounds that activate adenylate cyclase or phospholipase C (or both) are shown at the bottom of the Fig. under the respective pathway. hPTH(1–84), human parathyroid hormone (1–84).

material, to develop a nasally or orally deliverable drug, and to design a third-generation, nonpeptide structure, that more than half of the PTH holoprotein and its PLCβ- and PKC-stimulating activity are unnecessary for building bone. It is even more encouraging with the development of the functionally simplest adenylate cyclase-stimulating fragment, hPTH(1–31)NH_2, which is as osteogenically effective as the larger, dual-signalling hPTH(1–84) and hPTH(1–34), but has none of their primary Ca^{2+}- and PKC-induced actions.

At present, the major drawback of PTH therapy is that the hormone, or its fragments, must be injected subcutaneously, which might make it difficult to obtain patient compliance over a period of several years. However, there are some very preliminary indications that hPTH(1–31)NH_2, like calcitonin[6,40], can get into the blood through nasal blood vessels, but it is not yet known whether nasally administered hPTH(1–31)NH_2 stimulates bone growth in ovariectomized rats. So far, hPTH(1–31)NH_2 appears to be the smallest of the potently osteogenic unmodified PTH fragments, and it has been found[36] that the next smallest fragment, hPTH(1–30)NH_2, does not stimulate bone growth in the ovariectomized rat model although it stimulates adenylate cyclase in cultured rat osteoblasts as effectively as the larger peptides[44]. It remains to be determined whether the inability of hPTH(1–30)NH_2 to stimulate bone growth is due to an inability of the fragment to reach the circulation when injected subcutaneously. Overcoming this problem with hPTH(1–30)NH_2 could be an important step in the development of even smaller anabolic fragments and ultimately nonpeptide mimetics for noninjectable administration.

Concluding remarks

An effective treatment for osteoporosis may consist of daily (or hopefully even fewer) subcutaneous injections or nasal administration of a low dose of a small adenylate cyclase-stimulating PTH fragment, such as hPTH(1–31)NH_2, to build up the remaining trabecular bone, followed by an oral or transdermal osteoclast suppressor (oestradiol-17β) to prevent the new bone from being promptly resorbed. Since hPTH(1–31)NH_2 only activates adenylate cyclase, it should have fewer side-effects than the double-signalling hPTH(1–84) parent. The potential uses for a small PTH fragment extend beyond osteoporosis therapy, especially if it can be given in some way other than by injection. It should also be able to promote fracture healing, reverse the bone loss resulting from prolonged immobilization for various reasons, and be a type of 'chemical gravity' to maintain bone density during prolonged space missions.

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Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release

Rebecca Sitsapesan, Stephen J. McGarry and Alan J. Williams

In a variety of vertebrate and invertebrate tissues the ryanodine-sensitive Ca^{2+} channel is the pathway for Ca^{2+} release from intracellular stores. The mechanism for activation of the ryanodine receptor–channel complex appears to depend both on the ryanodine receptor isoform and the cell type. In addition, a complex combination of endogenous intracellular compounds regulates channel gating. In this article, **Rebecca Sitsapesan, Stephen McGarry and Alan Williams** review the mechanisms involved in cyclic ADP-ribose (cADPR)-induced Ca^{2+} release and discuss the likelihood that cADPR-activated Ca^{2+} release is mediated by one of the recognized isoforms of the ryanodine receptor–Ca^{2+} channel complex.

The ryanodine receptor–Ca^{2+} channel complex is a homotetramer, in which each monomer has a molecular weight of approximately 550 kDa (Ref. 1). Following the cloning and sequencing of the cDNA of the mammalian skeletal muscle ryanodine receptor (RY_1 receptor)[2], two other mammalian isoforms of the protein have been identified and their amino acid sequence deduced from their cloned cDNA. The ryanodine RY_2 receptor, which shows 66% identity with the RY_1 receptor, is expressed in cardiac muscle and brain, while the RY_3 receptor, which shows 67% identity with the RY_1 receptor, is expressed in the brain and at low levels in many other tissues[1,3]. The sarcoplasmic reticulum (SR) membranes of bird, fish and amphibian skeletal muscles contain two isoforms of ryanodine receptor[1,4,5]; the α isoform of the bullfrog receptor shows 80% identity with the RY_1 receptor, while the β isoform shows 86% identity with the RY_3 receptor[1]. Screening of an insect genomic library has identified an analogue of the mammalian ryanodine receptor gene that displays 45% identity with the RY_1 receptor[6].

Ryanodine disrupts Ca^{2+} handling in cells expressing well-defined ryanodine receptor isoforms (such as mammalian skeletal and cardiac muscle[7]) and is known to modify Ca^{2+} handling in other cell types, in which information on the identity of the isoform of ryanodine receptor or the levels of expression (or both) of the receptor is either less well-defined or absent. In the latter case, the pharmacological characterization of the putative ryanodine receptor usually reflects measurements of alterations in ryanodine-sensitive Ca^{2+} release in intact cells or cell homogenates. Detailed characterization of the function of the ryanodine-sensitive Ca^{2+} channel has largely been carried out using RY_1 (from mammalian skeletal muscle) and RY_2 receptors (from mammalian cardiac muscle). Studies involving the measurement of [^{3}H]ryanodine binding to, and Ca^{2+} efflux from, isolated SR membrane vesicles together with monitoring parameters of single-channel gating and conduction following the incorporation of either intact SR membrane vesicles or isolated receptor proteins into phospholipid bilayers, have identified a number of functional characteristics that are shared by these two isoforms and also some significant differences.

Functional consequences of ligand interaction with RY_1 and RY_2 receptors

A diverse array of ligands are known to modulate the function of ryanodine-sensitive Ca^{2+} channels[8]. The vast majority of these agents act at sites located on the cytosolic side of the Ca^{2+} channel (Fig. 1). To date, annexin VI (Ref. 9) and Ca^{2+} (Refs 10, 11) are the only agents thought to act at the luminal side of the channel. Although the mechanisms governing activation of RY_1 and RY_2 receptors *in situ* are thought to be different (mechanical coupling of RY_1 receptors in skeletal muscle, and Ca^{2+}-induced Ca^{2+} release for RY_2 receptors in cardiac muscle), following incorporation of the receptor into planar bilayers both isoforms are regulated by cytosolic Ca^{2+}. All ligands

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