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Receptor mechanisms and their role in drug interactions: effects of anaesthetics on G-protein-activated intracellular signalling pathways

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Different types of receptor-mediated mechanism play a key role in cellular transmembrane communication. The majority of plasma membrane receptors mediate the effects of neurotransmitters and hormones through activation of GTP-binding proteins (G-proteins). Coupling of the activated receptor to a G-protein initiates (occasionally inhibits) a cascade of enzyme-catalysed reactions leading to the production of one or more second messengers, eventually leading to the physiological response. The most commonly known cascades are the phosphoinositide and the cAMP route. This paper will describe the key concepts of G-protein-mediated signalling of both cascades and introduce the concept of 'cross-talk'. Further, the effects of anaesthetics on the intracellular components of these signalling pathways will be reviewed.

Key words: anaesthetics; halothane; isoflurane; G-protein; phospholipase C; inositol-phosphate; calcium handling; protein kinase C; protein kinase A; cyclic AMP.

During the last two decades, medical science has gained increasing knowledge of the basic cellular mechanisms involved in the effects of drugs that produce the state of general anaesthesia. Research on the mechanism of anaesthesia initially focused on the putative role of neuronal ion channels (for review see: Franks and Lieb, 1994). Recently, this field has expanded further and much research has been directed at interactions at the cellular level.

Interaction of an agonist with a cell-surface receptor leads to amplification of the signal through an intracellular cascade of events, eventually

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leading to the cellular response. In combination with modern molecular biological techniques, the study of intracellular signalling has led to the discovery of a range of plasma membrane receptor subtypes that had so far escaped classical pharmacological techniques. Nowadays, three types of receptor-mediated pathway are discerned: receptor-operated ion-channels, G-protein-activated signalling pathways, and the tyrosine kinase pathways. The effects of anaesthetics on receptor-operated ion channels have been studied extensively, whereas studies involving the other pathways are far less common. The purpose of this review is to describe different aspects of G-protein-activated signalling of different signal transduction pathways, including the concept of 'cross-talk'. Further, the effects of anaesthetics on components of the G-protein transduction pathway will be discussed.

CELLULAR SIGNALLING MECHANISMS

Signalling from plasma membrane surface receptors can be subdivided into the two main groups described below.

Receptor-operated ion channels

The first group consists of membrane-localized receptor proteins on which the agonist interacts with its outwardly directed recognition site. The interaction induces a conformational change in the protein in such a way that a transmembrane ion channel opens. In its open state, the ion channel allows the flow of particular ions, driven by their electrochemical gradient. Usually, plasma membrane receptors of this type consist of transmembrane pentamers, arranged in a circle with a central pore and operating in the msec range. The skeletal muscle type nicotinic acetylcholine receptor represents the classical model for receptors of this class, which also includes nervous tissue nicotinic acetylcholine receptor, the excitatory amino acid receptors (e.g. specific types of receptors to N-methyl-D-aspartate (NMDA), Amino-methyl-propionic acid (AMPA), kainate) and the inhibitory glycine and GABA-A receptors from the brain.

However, receptor-operated ion channels are not confined to the plasma membrane but exist also on intracellular compartments. Intracellular receptor-operated ion channels are activated by intracellular agonists produced by G-protein-activated signalling cascades. Examples are $InsP_3$ and ryanodine receptors, which mediate the release of Ca^{2+} from internal stores (see below).

Enzyme-activating receptors

Another mechanism to convey information from an extracellular first messenger to the inside of the cell uses second-messenger system(s) for transmembrane communication. Interaction of an agonist with a transmembrane enzyme-activating receptor leads to activation (occasionally inactivation) of a membrane-associated enzyme or to opening or closing of

an ion channel that is separate from the receptor complex. Signal transduction employing these mechanisms is much slower than receptor-operated ion channels (>100 msec to minutes range).

Within the subclass of G-protein-coupled receptors, the initial link in the chain of events involves coupling of the activated receptor to a GTP-binding protein (G-protein). The G-protein in turn modulates an ion channel and/or a plasma-membrane-bound enzyme ('effector protein'). The activated effector protein catalyses the formation of small signal molecules called second messengers. Second messengers may directly produce cellular effects, for example by binding to ion channels, or initiate the next step of the signalling cascade, eventually leading to an overall cellular effect. The receptor, the effector protein, its cellular target(s) and any intermediate molecule(s) are referred to as the 'cellular transduction pathway'.

Tyrosine kinase receptors constitute another subclass of transmembrane signalling receptors and consist mainly of receptors for growth factors, including insulin. Such receptors couple directly to tyrosine kinase, which, in turn, activates a cascade of protein–protein interactions and tyrosine phosphorylations. This cascade will eventually lead to activation of transcription factors, thus influencing cell division and differentiation.

G-PROTEIN ACTIVATED SIGNALLING

G-protein-coupled receptors

Surface receptors that couple to G-proteins, although different in their relationship to the particular agonist, are members of the superfamily of seven transmembrane receptors (7 TM) or 'serpentine receptors'. The seven α-helices of their approximately 500 amino-acid-long single chain, crossing the membrane from side to side, show a high degree of homology in all G-protein-coupled receptors. The binding site to the agonist is usually formed by a hydrophilic cavity at the extracellular surface ends of one or more transmembrane segments (Donnelly et al, 1989; Hulme et al, 1990). Most G-protein-coupling receptors desensitize quickly on prolonged exposure to agonists. The process consists essentially of steric hindering of the GTP-binding site by specific receptor kinases, enzymes that phosphorylate specific amino acids such as tyrosine, serine or threonine (Lefkovitz, 1993). This induces endocytosis of the receptor, which is agonist-independent. The reversible character of the process (receptor recycling) guarantees rapid re-establishment of the responsiveness. Non-receptor kinases activated by second-messenger pathways, such as protein kinase A and C (PKA; PKC), also modulate receptor desensitization through phosphorylation of the receptor (Clarke et al, 1989; Post and Dawson, 1992). Thus, activation of receptor-specific kinases always represents a form of homologous desensitization. On the other hand, inactivation of a specific receptor caused by another receptor activating PKA or PKC represents a case of heterologous desensitization and could be classified as 'cross-talk' between the two signal transduction routes.

Short conserved stretches in the third cytoplasmic loop of the receptor and in the carboxyl-terminal tail seem to be of particular importance for the specificity of G-protein coupling (O'Dowd et al, 1988). Linkage of the carboxy-terminal part to the plasma membrane further optimizes G-protein coupling to the receptor. Thus, laboratory-mutated 7 TM receptors lacking the above parts of the protein lose affinity and specificity for G-proteins. Mutations in G-protein-coupled receptor genes leading to loss of function cause sporadic inherited diseases such as retinitis pigmentosa, congenital bleeding due to thromboxane A_2 deficiency, Hirschsprung's disease and forms of colour and night blindness (reviewed by Spiegel, 1995). Nature has also accomplished the opposite. Specific point mutations in receptor regions that couple to the G-protein lead to continuous activation of its G-protein in the absence of an agonist. The first of such 'constitutively active receptors' were identified as causes for familiar premature puberty (LH receptor; Shenker et al, 1993) and hyperthyroidism (thyrotropin receptor; Parma et al, 1993).

GTP-binding proteins (G-proteins)

G-proteins transfer the signal from an activated 7TM receptor to an effector enzyme (Figure 1). G-proteins consist of three different subunits: α, and a

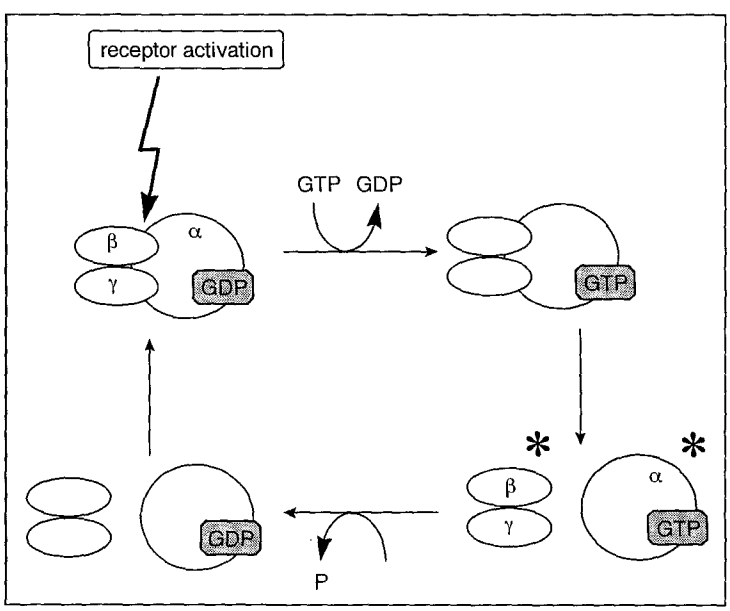

Figure 1. Schematic representation of the life cycle of a G-protein. Upon receptor stimulation (top left), the G-protein binds to the receptor and binds GTP, releasing the bound GDP. Subsequently the α-subunit dissociates from the βγ subunit, activating both the αGTP and βγ subunit (*, bottom right). Due to intrinsic GTPase activity of the α-subunit the bound GTP is hydrolysed to GDP (bottom left), increasing the affinity of the α subunit for βγ subunits. After reconstitution of α and βγ subunits, the G-protein is available for transduction of signals again.

complex of β and γ. On coupling to the activated receptor, the GDP bound to the α-subunit of the G-protein is replaced by GTP. Moreover, the α-subunit dissociates from the βγ complex. Both subunits can regulate (different) effector enzymes (Birnbaumer and Birnbaumer, 1995). The process terminates as a result of the intrinsic GTPase activity of the α-subunit, leading to inactive αGDP coupling with the βγ complex again.

Traditionally, classification of receptors in pharmacology is based on ligand binding activity. A different approach emerged from molecular biological techniques, and nowadays classification of receptors in subtypes is often based on amino-acid sequences of receptor sites. About 20 mammalian isoforms of G-protein α-subunit are currently known; these are divided into four major classes on the basis of amino-acid homology; each class couples to different effectors (Table 1) (Neer, 1995). Although about half in number, multiple β- and γ-subunit isoforms have also been identified (Neer, 1995). While βγ subunits have long been regarded as mere chaperones of the α-subunit, recent studies have shown that they also regulate physiological functions, for example, through activation of receptor kinases, phospholipase Cγ, subtypes of adenylate cyclase and plasma membrane K^+ and Ca^{2+} ion channels. The large variety in G-protein subunit isoforms may serve to ensure a certain selectivity of coupling to receptors and effector enzymes. It should be noted, however, that it is not uncommon for a specific type of receptor to activate different G-proteins, thus activating multiple cellular signalling pathways (Henning et al, 1993a,b, 1996). On the other hand, several studies have indicated that cells possess some degree of compartmentalization to control dynamics of the binding of G-protein to receptors (Graeser and Neubig, 1993; Leiber et al, 1993), given the excess of G-proteins over receptors and effectors. Two toxins, pertussis toxin and cholera toxin, which interact with specific G-proteins have been identified and are commonly used in research. Both toxins work in a similar direction: pertussis toxin (PTx) irreversibly inhibits G_i-proteins, while cholera toxin activates G_s, both resulting in an increased activation of adenylate cyclase.

Table 1. Classes of G-protein and their signalling routes*.

Class	Members	Major signalling routes	Receptor examples
α_s	α_s, α_{olf}	Adenylate cyclase ↑	β-Adrenergic, adenosine-A2, Dopamine D1,5, GABA-B
α_i	α_{i1-3}, α_o, α_{t1-2}, α_{gust}, α_z	Adenylate cyclase ↓ Regulate K^+ and Ca^{2+} channels Activate cGMP phosphodiesterase	Opioid, adenosine-A1, α2 Adrenergic, dopamine D2,3,4, Muscarinic M_2, M_4
α_q	α_q, α_{11}, α_{14-16}	Activate phospholipase C	α_1-adrenergic histaminic Vasopressine, muscarinic M_1, M_3, M_5
α_{12}	α_{12}, α_{13}	Regulate Na^+/K^+ exchange	

* Classes are named after their α subunit, for example, α_s belongs to the class of G_s proteins. For details see, for example, Clapham and Neer (1993).

By now more than 200 receptors coupling to G-proteins have been identified. Therefore, G-proteins are probably involved in virtually every physiological process, from maintaining cellular homeostasis to regulation at organ- and even whole-body level. Many neurotransmitters relevant to anaesthesia, including acetylcholine (muscarinic receptors), dopamine, noradrenaline, serotonin, adenosine and neuropeptides, function through G-protein-coupled receptors. This paper will deal with the two classical effector pathways of G-proteins: the phospho-inositide and the cAMP pathway.

Anaesthetic effects

Recently, several studies have shown significant effects of anaesthetics in clinically relevant concentrations, which might be attributed to interference at the G-protein level of specific receptors.

α_2-Adrenoceptor

Hypnosis, analgesia and reduction of anaesthetic requirements have been demonstrated in clinical and laboratory studies with the drugs dexmedetomidine and clonidine. These effects are mediated through α_2-adrenoceptors, which are widely distributed throughout the nervous system. Studies in rats employing the response to α_2-adrenoceptor activation following intrathecal and intra-cerebroventricular injections of pertussis toxin (PTx) demonstrated a role for PTx-sensitive G_i-protein-coupled α_2-adrenoceptors in both hypnosis (Doze et al, 1990) and spinal analgesia (Hayashi et al, 1995). Data from these studies suggest that hypnotic and analgesic effects of the α_2-adrenoceptor agonist dexmedetomidine are transduced via PTx-sensitive G-proteins. Hypnotic effects seem to be localized in the locus coeruleus and the analgesic effect at the level of the spinal cord. Moreover, the clinically well known MAC sparing effect of dexmedetomidine did not change by inhibition of G_i-protein function. In an in vitro study using isolated rat middle cerebral artery it was concluded that α_2-adrenoceptor-mediated dilatation was mediated through PTx-sensitive G-proteins (Bryan et al, 1996), suggesting a role for α_2-agonists in cerebral blood flow regulation.

Muscarinic receptor

Muscarinic receptors are distributed widely in the CNS. They are involved in aspects of higher cerebral functioning as the level of consciousness, learning, memory and pain and are thus of interest for anaesthesiology (for review, see Durieux, 1996).

Several studies showed interaction of muscarinic G-protein-coupled receptors and volatile anaesthetics. Binding studies of high-affinity muscarinic agonist after halothane (Dennison et al, 1987) or enflurane and isoflurane (Anthony et al, 1989) suggested that inhibition of G-protein was the result of stabilization of the receptor G-protein complex or of interference with the GDP–GTP exchange. Later functional studies in *Xenopus*

oocytes supported these findings. Signalling through muscarinic receptors, in a model using *Xenopus* oocytes expressing mouse brain mRNA, was inhibited by enflurane (Lin et al, 1993). Similar results were obtained for cloned rat M_1-muscarinic receptor and halothane in the same model (Durieux, 1995). In both studies the site of action was not downstream from the G-protein. In contrast, isoflurane did not inhibit muscarinic signalling in the same model (Durieux, 1995). Interestingly, in this oocyte model, ketamine inhibits muscarinic signalling in a non-stereoselective fashion (Durieux and Nietgen, 1997). Although the S(+) form of ketamine is approximately three times as potent in patients compared to the R(−) form (White et al, 1985), the observed equal sensitivity of M_1 muscarinic receptor-coupled G-proteins to S(+) and R(−) ketamine does not exclude that the anaesthetic mechanism of ketamine action includes G-proteins as targets.

Lysophosphatidic acid receptor (LPA receptor)

Lysophosphatide (LPA) is a member of a novel group of lipid mediators involved in different biological effects such as smooth muscle contraction, cellular proliferation and platelet aggregation. These effects seem to be regulated through specific G-protein-coupled LPA receptors, involved in actin stress fibre re-organization (Ridley and Hall, 1992). Prostaglandins and platelet-activating factor are representatives of other substances mediated through LPA receptors. Their lipophilicity makes the receptor a candidate for interaction with propofol and volatile anaesthetics. A study in *Xenopus laevis* oocytes, which endogenously express LPA receptors, showed that clinically relevant concentrations of propofol inhibited LPA signalling at the level of the receptor or its associated G-protein (Rossi et al, 1996). Interestingly, a subsequent study of the same group produced different results for halothane and isoflurane. Halothane depressed LPA signalling in a concentration-dependent manner, but isoflurane had no effect at comparable clinically relevant concentrations (Chan and Durieux, 1997). This study also demonstrated that the site of action was not downstream of the G-protein in the coupled transduction pathway. Some of the side-effects of anaesthetics may be attributed to interference with LPA receptors.

β-Adrenoceptor

Halothane anaesthesia has been related to an increased tendency to develop catecholamine-induced arrhythmias in animal experiments (Hayashi et al, 1991) and human patients (Johnston et al, 1976). These effects have been related to interference with G-protein function. In a study using preparations of human myocardial tissue obtained from terminally failing hearts, halothane has been found to stimulate adenylate cyclase activity. Adenylate cyclase activity is under dual control of stimulatory (G_s) and inhibitory (G_i) G-proteins (Gilman, 1984). The results of the study were explained by inhibition of the G_i of the β-adrenoceptor, probably through direct interaction at the α- or βγ-subunits (Böhm et al, 1994; Schmidt et al, 1995) and not by a hypothetical effect on the G_s protein. In vitro studies of

human myocardial tissue (Böhm et al, 1993) and rat left ventricular papillary muscle (Hanouz et al, 1997) demonstrated that halothane at clinically relevant concentrations potentiates the positive inotropic response of β-adrenoceptor stimulation. This increased contractility was explained by inhibition of G_i. Therefore, the mechanisms through which halothane sensitizes for arrythmias and enhances positive inotropic effects of catecholamines are probably mediated through inhibition of G_i-proteins.

Opioid receptors

Traditionally, opioid receptors are thought to couple to inhibitory G-proteins (G_i) and exert their action through inhibition of the adenylate cyclase pathway, thus decreasing cAMP levels. However, recently a variety of additional—sometimes secondary—effects on other signal transduction pathways were described, mainly in cultured cells. Among these are the enhancement of Ca^{2+} influx and the activation of phospholipase C (PLC) through the βγ subunits of the G_i and subsequent production of $InsP_3$, release of Ca^{2+} from internal stores and activation of protein kinase C (PKC). Therefore, opioids may influence many other cellular signalling pathways through cross-talk mechanisms. However, no direct effects of opioids on intracellular proteins have been described. For further reading, the reader is referred to a comprehensive review of the cellular effects of opioids by Smart and Lambert (1996).

G-PROTEIN EFFECTORS: PHOSPHO-INOSITIDE METABOLISM

Many extracellular ligands stimulate receptors whose G-protein couples to a membrane-bound enzyme called phospholipase C (PLC) acting on a specific phosphatidylinositol derivative (Figure 2). The phosphatidyl-inositols are a class of inner-leaflet plasma membrane lipids. Activation of PLC causes hydrolysis of the 4,5-bisphosphate form (PIP_2) to produce two second messengers: water-soluble inositol (1,4,5)-trisphosphate ($InsP_3$) and lipophilic diacylglycerol (DAG). $InsP_3$ acts on intracellular Ca^{2+} stores, whereas DAG activates protein kinase C (PKC). Of the several classes of PLC, the PLCγ class of isoenzymes is stimulated by G-proteins via specific α and/or βγ subunits.

$InsP_3$ formation and intracellular Ca^{2+} release

The $InsP_3$-activated release of intracellular Ca^{2+} serves mainly to mediate acute effects of the PLC route. Upon diffusion, $InsP_3$ stimulates a specific intracellular Ca^{2+} release channel of the endoplasmic reticulum, the $InsP_3$ receptor, which is a hetero-pentameric protein of which subunits come in three isoforms. In addition to the release of Ca^{2+} from internal stores, the emptying of $InsP_3$-sensitive Ca^{2+} stores itself causes an influx of Ca^{2+} across the plasma membrane ('capacitive Ca^{2+} entry') (Putney, 1986).

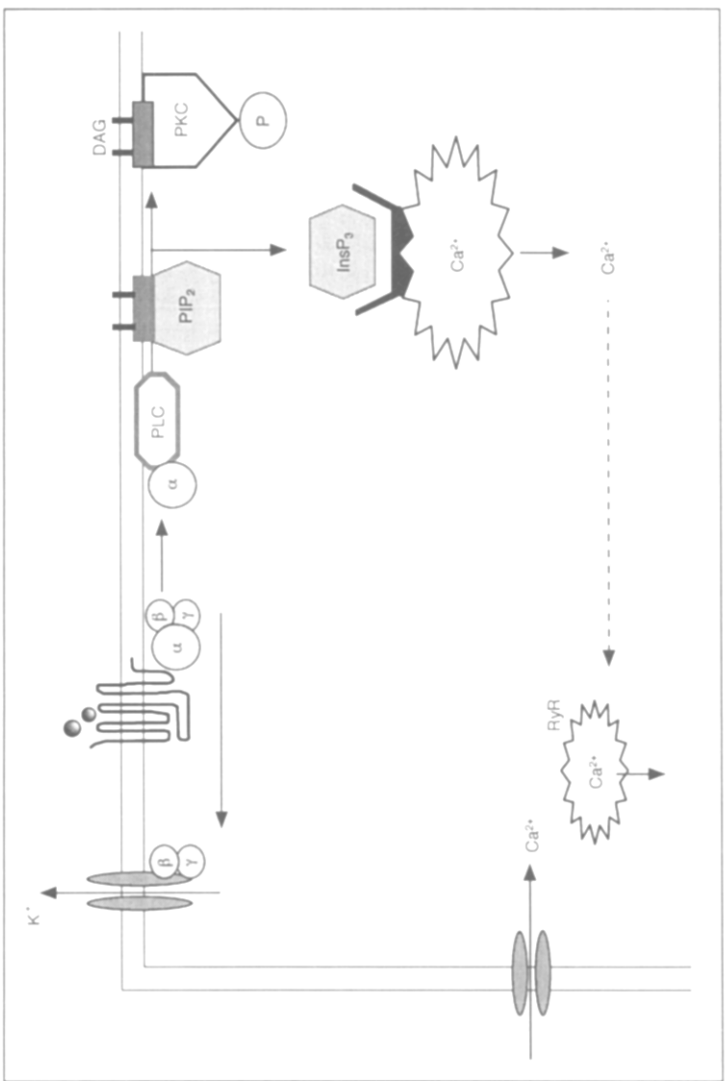

Figure 2. Schematic representation of the phospholipase C (PLC) pathway. Agonist stimulation of the transmembrane receptor activates the G-protein, of which the α-subunit binds to PLC. PLC induces hydrolysis of the membrane phospholipid PIP_2 into $InsP_3$ ('hydrophilic head') and diacylglycerol (DAG) ('lipophilic tail'). $InsP_3$ induces the release of Ca^{2+} from the $InsP_3$-sensitive intracellular store, while DAG activates protein kinase C (PKC). Ryanodine receptors (RyR) are activated by an increase in intracellular Ca^{2+}, thus releasing Ca^{2+} from the ryanodine-sensitive stores secondary to $InsP_3$-activated release of Ca^{2+} from internal stores and/or Ca^{2+} influx via the plasma membrane (bottom left). The G-protein's βγ-subunit stimulates a K^+ channel (top left). See text for more details.

Phosphorylation of $InsP_3$ receptors by PKA and PKC has been reported to modulate their function (Ferris et al, 1991). Increase in the intracellular Ca^{2+} may—depending on the tissue—activate cellular functions such as, for example, contraction, secretion or firing frequency. However, increase in Ca^{2+} may also indirectly modulate cellular effects through (in)activation of Ca^{2+}-sensitive proteins, including PKC (see below).

In many types of cell a second type of intracellular calcium store ('ryanodine stores') exist next to $InsP_3$-sensitive Ca^{2+} stores. The ryanodine store mediates a release of intracellular calcium in response to increased calcium concentrations in the vicinity of the ryanodine receptor ('calcium-induced Ca^{2+} release'). Thus, induction of $InsP_3$-mediated release of Ca^{2+} from internal stores may induce release of Ca^{2+} from ryanodine-sensitive stores (Figure 2). In reverse, calcium release from ryanodine-sensitive stores can also influence release of Ca^{2+} from $InsP_3$-sensitive stores, as many of the catalytic proteins of the PLC cascade are modulated by the intracellular Ca^{2+} concentration (including $InsP_3$ receptors). Such complex interactions between Ca^{2+} released from $InsP_3$- and ryanodine-sensitive Ca^{2+} stores and the calcium entering via plasma membrane ion channels are described in spatio-temporal models involving 'sparks' or 'blips' of calcium giving rise to calcium 'puffs', which, in turn, generate Ca^{2+} 'waves'.

Anaesthetic effects on $InsP_3$ and Ca^{2+}

A series of interactions between the PLC cascade and halothane have been reported. In the absence of receptor activation, halothane increased $InsP_3$ formation in turkey erythrocytes through stimulation of PLC (Rooney et al, 1993) and $InsP_3$ levels in human neuroblastoma cells (Smart et al, 1994). Halothane was shown to inhibit receptor-activated $InsP_3$ production in rat aorta A7r5 cells (Sill et al, 1991), human platelets (Kohro and Yamakage, 1996) and in turkey erythrocytes (Rooney et al, 1993). By contrast, in human neuroblastoma cells, halothane stimulated receptor-activated $InsP_3$ formation (Smart et al, 1994). Further, effects of halothane on the $InsP_3$-sensitive store have been reported. Halothane enhances the spontaneous leak from these stores, thus depleting them of Ca^{2+} (Sill et al, 1991; Evers and Hossain, 1994; Kohro and Yamakage, 1996). Finally, halothane has been shown to inhibit $InsP_3$ diffusion to neighbouring cells through gap junctions (Sanderson et al, 1990).

Studies examining isoflurane often arrive at similar conclusions. Isoflurane inhibits $InsP_3$ formation and release from $InsP_3$-sensitive stores in A7r5 cells (Sill et al, 1993), increases spontaneous leak from the store (Evers and Hossain, 1994) and increases basal and receptor-activated $InsP_3$ production in human neuroblastoma cells (Smart et al, 1994). Differences with halothane were observed in turkey erythrocytes in which isoflurane had no effect on $InsP_3$ production and internal Ca^{2+} after agonist stimulation (Kohro and Yamakage, 1996). A study examining enflurane in *Xenopus* oocytes demonstrated inhibition of agonist- and GTP-activated ion currents, but not of currents evoked by intracellular administration of $InsP_3$, suggesting that enflurane inhibits $InsP_3$ signalling at the level of G-protein or PLC.

Besides their action on $InsP_3$-sensitive stores, halothane, isoflurane and enflurane were reported to release Ca^{2+} from ryanodine sensitive stores in permeabilized rat mesenteric artery (Akata and Boyle, 1995, 1996).

Ketamine has been reported to inhibit the PLC pathway stimulated by agonists (Ratz et al, 1993; Sato et al, 1997), although no effect was observed in rat liver (Shibata et al, 1993). Molecular targets have not been identified; however, it is unlikely that ketamine impairs the function of the $InsP_3$-sensitive Ca^{2+} store (Kanmura et al, 1996), but rather decreases $InsP_3$ levels. There are limited data on the effects of other intravenous anaesthetics on $InsP_3$ signalling. Fentanyl (Makita et al, 1994; Shibata et al, 1995) and diazepam (Makita et al, 1994) inhibit noradrenaline-induced $InsP_1$ formation in rat aorta and/or cortex, but were without effect in rat liver (Shibata et al, 1993). The barbiturates thiopentone (Shibata et al, 1995) and thiamylal (Shibata et al, 1993) were reported to stimulate noradrenaline-mediated $InsP_1$ production. In neuroblastoma, thiopentone was reported to directly inhibit agonist-evoked Ca^{2+} release from $InsP_3$-sensitive stores, as it did not influence $InsP_3$ levels (Lambert et al, 1996). Finally, propofol was found to inhibit $InsP_3$ production in aorta A10 cells (Xuan and Glass, 1996), which might be caused by a decrease in PLC activity due to propofol inhibiting Ca^{2+} entry through L-type Ca^{2+} channels (Lambert et al, 1996; Xuan and Glass, 1996).

All studies that examined the action of anaesthetics on capacitive Ca^{2+} entry (calcium entry via the plasma membrane induced by emptying of the intracellular stores) report a lack of action of anaesthetics on this phenomenon (Sill et al, 1993; Kanmura et al, 1996; Lambert et al, 1996; Xuan and Glass, 1996). This is a remarkable finding in view of the many effects on other types of plasma membrane ion channels.

By and large, it appears that volatile anaesthetics exhibit an inhibitory action on the PLC/Ca^{2+} pathway. Volatile anaesthetics seem to exert profound effects on intracellular stores, i.e. enhancement of spontaneous leak from the $InsP_3$-store as well as Ca^{2+} release from the ryanodine-sensitive store. Although it is unclear whether these processes are independent of each other, both will lead to an increase in intracellular Ca^{2+} and depletion of the store. It is unclear to what extent other cellular effects, such as modulation of $InsP_3$ formation or activation of PLC, are secondary to the change in intracellular calcium.

The effects of the intravenous anaesthetics are less homogenous. Because of the limited number of studies on individual compounds, molecular targets are difficult to identify. Nevertheless, it seems highly likely that part of the effects are produced by 'cross-talk' mechanisms (e.g. fentanyl inhibiting noradrenaline response).

The DAG pathway

DAG is the major endogenous activator of the serine/threonine kinase protein kinase C (PKC). The twelve PKC isoforms known are divided into three groups: Ca^{2+}-sensitive, Ca^{2+}-insensitive and 'atypical'. PKC mediates

numerous biological functions, as phosphorylation of many cellular proteins activates or inhibits their function (Figure 2).

Anaesthetic effects on PKC

Protein kinase C (PKC) has been implicated as a potential target for the action of general anaesthetics in various studies. Actions of volatile anaesthetics (mainly halothane and isoflurane) on spontaneous activation of PKC and modulation of activated PKC have been characterized in vivo, in intact cells and in vitro. The results obtained by the majority of studies in vivo and in intact cells indicate an inhibitory action of volatile anaesthetics on PKC-mediated responses (Yamakage, 1992; Firestone et al, 1993; Saito et al, 1993; Araki et al, 1994; Loeb et al, 1994; Namba and Tsuchida, 1996; Park et al, 1996). Volatile anaesthetics have been reported to indirectly stimulate activated PKC through an increase in intracellular calcium in PC12 cells (Tas and Koschel, 1991), whereas another study reports the absence of effects of halothane and isoflurane on PKC-mediated responses (Ozhan et al, 1994). Care must be taken to interpret these studies as they represent different cells and tissues with specific PKC isoforms and their cellular distributions, and the studies also employ different time frames. Further, the conclusions drawn with respect to the action of volatile anaesthetics in these studies are based mainly on indirect evidence, such as volatile anaesthetics attenuating the response to phorbol esters. Nevertheless, the number of studies indicating an inhibitory action of volatile anaesthetics on PKC remarkably outweighs the others.

In response to the above reports, a number of studies have attempted to characterize the effect of volatile anaesthetics on PKC by in vitro analysis. Both stimulatory (Hemmings and Adamo, 1994) and inhibitory (Slater et al, 1993) effects of volatile anaesthetics on purified brain PKC were observed, the difference being explained by co-factors in the assay medium. Later studies, employing artificial or endogenous lipid bilayer preparations, show more consensus by differentiating between effects of volatile anaesthetics on cytosolic and membrane-bound PKC (Hemmings et al, 1995; Hemmings and Adamo, 1996, 1997; Kumar et al, 1997; Slater et al, 1997). It seems that volatile anaesthetics exhibit two distinct actions: (1) activation of cytosolic PKC, and (2) translocation of cytosolic PKC to membranes, followed by down-regulation (see also Yamakage, 1992). These findings suggest that the latter process, explaining the inhibitory action of volatile anaesthetics on PKC-mediated responses observed in the majority of cellular and in vivo studies, represents the clinically relevant action of the volatile anaesthetics.

In many of the above studies the effects of halothane were compared with isoflurane; in general no major differences were found. The actions of a limited series of intravenous anaesthetics on PKC were studied in vitro only. Propofol has been reported to enhance PKC activity in a manner similar to that of halothane (Hemmings and Adamo, 1994; Hemmings et al, 1995), while barbiturates have been reported to inhibit PKC by competing for the DAG domain (Mikawa et al, 1990). The effects of the above

anaesthetics on PKC have been observed at concentrations corresponding to the moderate to high clinical range. Therefore, it is conceivable that effects on PKC modulate the anaesthetic effects of these compounds.

G-PROTEIN EFFECTORS: cAMP PATHWAY

The cyclic AMP pathway

Stimulation of the effector enzyme adenylate cyclase generates the second messenger cAMP, which in turn activates protein kinase A (PKA: Figure 3). cAMP is degraded by phosphodiesterases. Several isoforms of adenylate cyclase and phosphodiesterases are known, some of which have specific tissue distributions. G-protein α-subunits coupling to adenylate cyclase may exert either of two actions: inhibitory or stimulatory (hence their family name convention G_i and G_s). There are separate sites for α_s and α_i subunits, but also for βγ subunits on adenylate cyclase (Taussig et al, 1994). The action of βγ subunits varies among different isoforms of adenylate cyclase (stimulation, inhibition, and no effect). It has now been firmly established that factors other than G-protein subunits modulate adenylate cyclase activity, including Ca^{2+} and PKC.

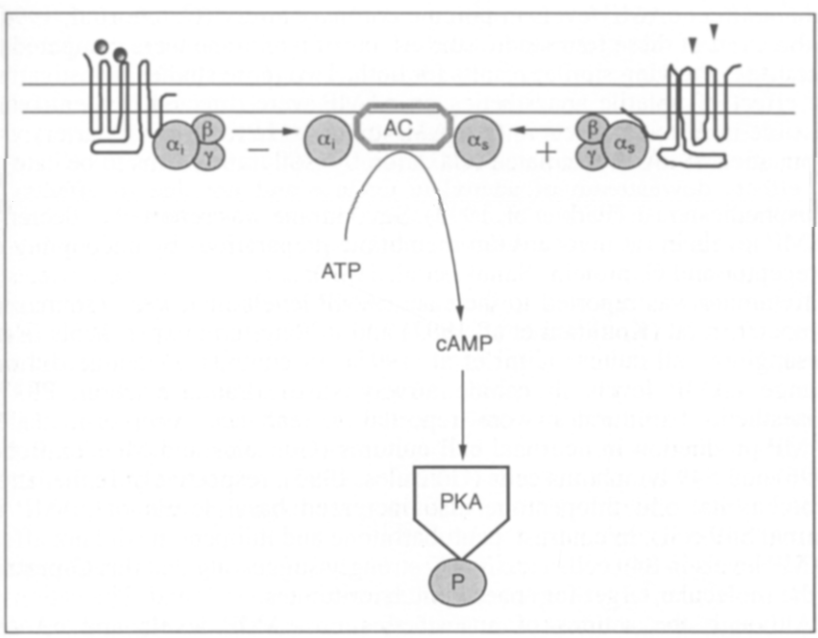

Figure 3. Schematic representation of the cyclic AMP (cAMP) pathway. The effector enzyme adenylate cyclase (AC) metabolizes ATP to cAMP, which, in turn, activates the protein kinase A (PKA). AC is activated by α_s subunits from a G_s-protein (right), or inhibited by α_i-subunits from a G_i-protein (left) upon activation of their respective receptors. See text for more details.

As in the case of PKC, many biological functions of PKA have been revealed, of which a few were mentioned above. Besides short-term effects of PKA through phosphorylation of cellular proteins, a long-term regulatory mechanism consists of regulation of transcription factors that bind to cAMP responsive elements (CRE) in the promoter regions of cAMP-inducible genes (Combe et al, 1986).

Anaesthetic effects

As the cAMP pathway represents a major signalling cascade involved in the relaxation of vascular and bronchial smooth muscles, the effects of many anaesthetics have been explored in animal in vitro models. Most often, this was accomplished by measurement of muscle relaxation after receptor-mediated pre-contraction, which was frequently accompanied by measurement of cAMP levels. Unfortunately, many of these studies produced conflicting results, and only few studies examined the level at which anaesthetics interfere in the cAMP cascade.

Of the volatile anaesthetics, halothane was reported to inhibit receptor-mediated increases in cAMP in adipocytes (Ohlson et al, 1997) and to inhibit cAMP-mediated ion currents in cultured bullfrog dorsal root neurones (Tokimasa et al, 1990). Halothane stimulates receptor-mediated cAMP production in canine tracheal smooth muscle (Yamakage, 1992), but did not affect cAMP levels in porcine coronary artery (Ozhan et al, 1994). In three out of these four studies the effects of isoflurane were compared to halothane, showing similar results for both. Two more studies investigating the effect of volatile anaesthetics on cAMP were directed at identifying possible molecular targets in the cAMP cascade. In rat coronary artery, the attenuation of cAMP mediated relaxation by isoflurane seems to be caused by effects downstream of adenylate cyclase and not due to effects on phosphodiesterase (Park et al, 1995). Sevoflurane was reported to decrease cAMP levels in rat myocardium membrane preparations by uncoupling of β-receptor and G_s protein (Sanuki et al, 1994).

Ketamine was reported to increase cAMP levels of lower oesophageal sphincter in rat (Kohjitani et al, 1997) and in long-term experiments in rat mesangium cell culture (Jimi et al, 1997). In contrast, ketamine did not change cAMP levels in canine airway strips (Pabelick et al, 1997). Anaesthetic barbiturates were reported to enhance receptor-mediated cAMP production in neuronal cell cultures (Gonzales and Mendez-Bobe, 1996) and S49 lymphoma cells (Gonzales, 1995), respectively. In the latter, pentobarbital and thiopentone also increased basal levels of cAMP in normal S49 cells. In contrast, pentobarbitone and thiopentone did not affect cAMP levels in S49 cells lacking G_s, strongly suggesting that this G-protein is the molecular target for anaesthetic barbiturates.

Although the actions of anaesthetics on cAMP levels and cAMP-mediated responses have been established in a variety of models, the molecular mechanisms are at large unknown. Apart from established mechanisms such as the actions on G_s, it is highly conceivable that actions of anaesthetics on the cAMP route may be produced by 'cross-talk' through

their modulation of other signalling cascades (notably the PLC pathway; see above). An example of this may be the reported increase in cAMP by halothane, which may be secondary to inhibition of the thrombin-induced PLC signalling in human platelets (Kohro and Yamakage, 1996).

CROSS-TALK AND NETWORKING

In the light of the above, it may not be surprising to learn that the PLC route and the cAMP pathway interact at various levels. Indeed, the effectors of the PLC pathway, Ca^{2+} and PKC have been reported to exert potent inhibitory and stimulatory effects on the adenylate cyclase pathway. Effects of Ca^{2+} and PKC have been described on the level of adenylate cyclase itself, but also by affecting modulators of adenylate cyclase (ranging from receptor kinases to phosphatases and proteases). Such interactions may be very complicated, for example, in mouse muscle C2C12 cells, activation of the PLC route through P_2 purinoceptor stimulation enhances cAMP formation elicited by adenosine receptors, but strongly inhibits cAMP formation activated by β_2 or ATP receptors (Figure 4; Henning et al, 1993a,b).

The opposite phenomenon, i.e. the cAMP pathway affecting the PLC route, has been the subject of many studies. Both the $InsP_3$/DAG metabolism and the regulation of Ca^{2+} homeostasis are affected by PKA. PKA-mediated phosphorylation of PLC, $InsP_3$ receptor subtypes and plasma membrane Ca^{2+} channels may serve as examples.

FINAL REMARKS

There is no doubt that new interaction points between signal transduction routes, and perhaps even new signalling cascades, will be discovered. Moreover, nearly all proteins involved in the signalling cascades as effector or modulator consist of multiple isoforms, which are often expressed cell type-specific. Because of the virtually unlimited possibilities of interaction, researchers in the field of cellular signalling are moving away from the concept of separated signalling cascades. Rather, cellular signalling must be viewed as a signalling network, in which receptors represent focal entry points. The ultimate response of a cell or tissue to a single stimulus will depend largely on the state of its signalling network. Cell- or tissue-specific expression patterns of proteins involved in signal transduction will set the boundaries of the overall response to an extracellular agonist. Their expression levels can be modulated through (long-term) activation of signalling pathways. On top of that, short-term regulation is accomplished by cross-talk mechanisms.

In theory, the action of anaesthetics may be produced by interaction with one protein of a single signalling cascade, the subsequent response being the result of the effect of this signalling cascade together with cross-talk mechanisms available in the given type of cell. This is likely to be true for many intravenous anaesthetics that interact with plasma membrane

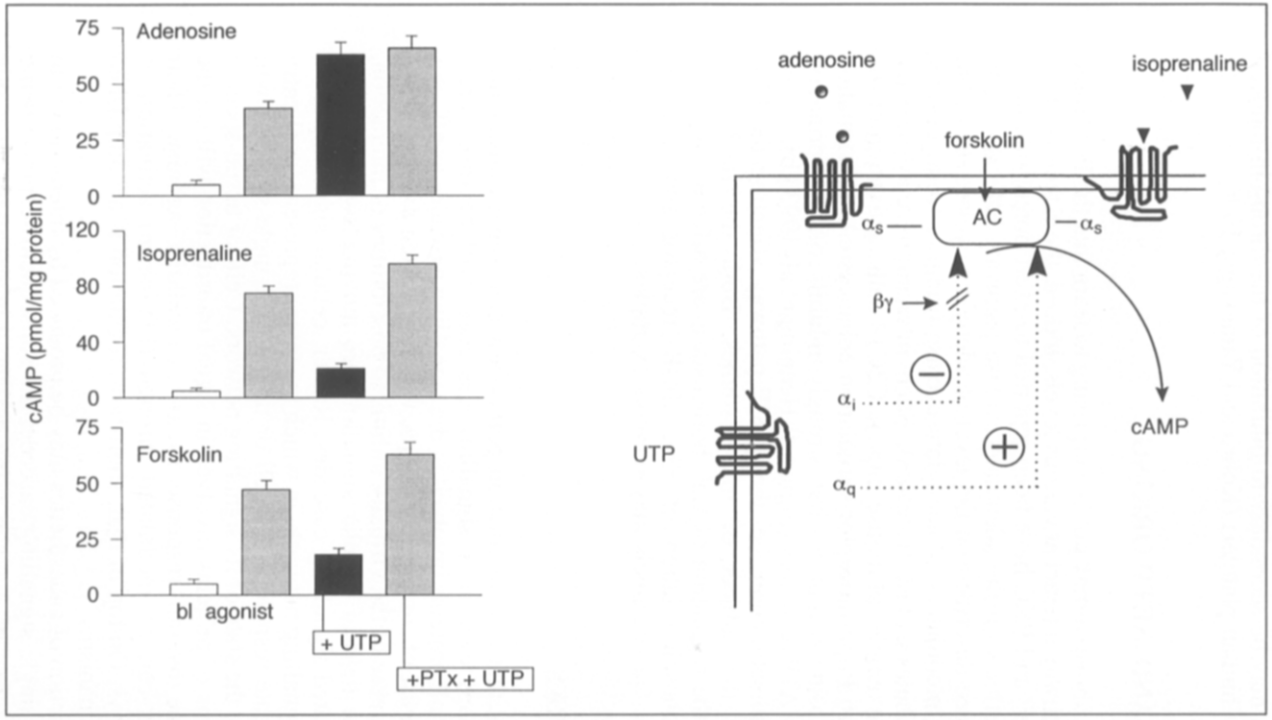

Figure 4. Experiments in mouse C2C12 myotubes demonstrating the cross-talk from the $InsP_3$ upon the cAMP pathway. Left panel: cAMP levels were measured by a radioligand binding technique in 6-day differentiated myotubes. The first bar (b1) indicates cAMP levels in the absence of the agonist. Differentiated cells were stimulated for 10 minutes by an agonist for the β_2 receptor (isoprenaline), adenosine A_2 receptor (adenosine) or by direct activation of adenylate cyclase (AC) by forskolin, and cAMP levels were measured (agonist; second bar). The third bar indicates cAMP levels obtained at treatment with these agonists in the presence of the P_{2U} purinoceptor agonist UTP, activating the phospholipase C (PLC) pathway through α_q and α_i G-protein subunits. Cross-talk from the PLC pathway enhances cAMP production elicited by adenosine, but strongly inhibits cAMP formation due to stimulation with isoprenaline or forskolin (third bar). The last bar shows that the inhibitory effect of the PLC pathway on cAMP formation is caused by α_i subunits as the inhibition is overcome completely by pre-treatment with PTx (12 hours). Right panel: schematic representation of the putative cross-talk mechanism: $\beta\gamma$ subunits of the adenosine receptor, but not of the β receptor, quench the α_i, thus preventing α_i-mediated inhibition of cAMP formation.

receptors, including opioids, benzodiazepines, barbiturates or α_2-agonists. Still, there may be large variations in drug response between individuals, depending on differences in the fundamental architecture of their signalling network ('genotype') and superimposed modulation by, for example, co-morbidity, stress and medication ('phenotype').

On the other hand, anaesthetics may interact with the signalling network at multiple points. This may be especially true for the volatile anaesthetics, whether one tends to accept or reject the lipid perturbation theory as explaining the mechanism of action of volatile anaesthetics. In the case of halothane, interaction with signalling pathways is confined mainly to inhibition of the PLC cascade. Thus, uncoupling of G-proteins, reduced production of $InsP_3$, enhancement of spontaneous leak from the $InsP_3$ store and brief activation of PKC, followed by desensitization, were reported at clinically relevant concentrations. Other volatile anaesthetics, mainly isoflurane, sometimes lacked the specific effect as described for halothane, but generally the effects of both compounds were similar. Although it is unclear whether the interference with the $InsP_3$ pathway contributes significantly to the state of general anaesthesia produced by volatile anaesthetics, it is conceivable that interaction at specific intracellular targets by the various anaesthetics results in differences with respect to the components of anaesthesia and/or its side-effect profile.

Up till now, very few experimental studies have addressed the effects of multiple anaesthetics in a cellular system, and their cross-talk mechanisms. To unravel these multiple points of interaction further, studies on organs or cells need to be conducted in co-ordination with studies on subcellular particles and isolated proteins, and vice versa. The results of such studies will undoubtedly increase our insight in cellular signalling, but may also lead to the understanding of the mechanisms underlying the effect and side-effects of anaesthetics, eventually facilitating the recognition or development of new effective and safer drugs for anaesthesia.

The cross-talk concept is already part of clinical anaesthesiology. Premedication and classical balanced anaesthesia may be regarded as successful attempts to influence the signalling network of target cells—whatever they may be—to produce desired levels of general anaesthesia at reduced risk.

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