# Activation of muscarinic $K^+$ current in guinea-pig atrial myocytes by sphingosine-1-phosphate

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1. Activation of muscarinic $K^+$ current ($I_{K(ACh)}$) by sphingosine-1-phosphate (Sph-1-P) was studied in isolated cultured guinea-pig atrial myocytes using whole-cell voltage clamp.

2. Sph-1-P caused activation of $I_{K(ACh)}$ with an $EC_{50}$ of 1·2 nM. The maximal current that could be activated by Sph-1-P amounted to about 90% of the $I_{K(ACh)}$ caused by a saturating concentration of acetylcholine (ACh, 10 μM). Sphingosine (1 μM), which can mimic the signalling effects of Sph-1-P in other cells, failed to cause measurable activation of $I_{K(ACh)}$.

3. $I_{K(ACh)}$ activation by Sph-1-P was completely suppressed in cells treated with pertussis toxin.

4. Desensitization of muscarinic receptors by pre-incubation of the cells with carbachol did not affect the response to Sph-1-P; likewise, pre-incubation of the cells with Sph-1-P resulted in a reduced sensitivity to the phospholipid but not to ACh. In contrast, pre-incubation with either Sph-1-P or a serum phospholipid previously described as activating atrial $I_{K(ACh)}$ resulted in reduced sensitivity to both phospholipids.

5. It is concluded that activation of $I_{K(ACh)}$ by Sph-1-P in atrial myocytes is induced by binding to a novel G protein-coupled phospholipid receptor.

Sphingosine and sphingosine-1-phosphate (Sph-1-P), metabolites of membrane sphingolipids, have been demonstrated to act as signalling substances in a number of different cell lines. Sph-1-P has been shown to release $Ca^{2+}$ from the endoplasmic reticulum (ER) by a mechanism independent of inositol trisphosphate ($InsP_3$) (Ghosh, Bian & Gill, 1994; Mattie, Brooker & Spiegel, 1994). In this regard its mechanism of action is distinctively different from that of lysophosphatidic acid, another phospholipid mediator, which, in various cells, causes $Ca^{2+}$ signalling via the $InsP_3$ pathway (Jalink, van Corven & Moolenaar, 1990; Durieux, Salafranca, Lynch & Moorman, 1992; Tigyi & Miledi, 1992). Apart from inducing $Ca^{2+}$ release, exogenously applied Sph-1-P has recently been demonstrated to cause a decrease in cellular cAMP levels and increases in inositol phosphate formation, cytoplasmic $Ca^{2+}$ concentration, mitogen-activated protein (MAP) kinase activity and accumulation of phosphatidic acid (PA) by activation of phospholipase D in swiss 3T3 fibroblasts (Goodemote, Mattie, Bergers & Spiegel, 1995; Wu, Spiegel & Sturgill, 1995). These signalling events induced by Sph-1-P, except for accumulation of PA, were attenuated by pertussis toxin (PTX), suggesting that a $G_i/G_o$ type of G protein is involved in some cellular signals caused by Sph-1-P. Both a putative Sph-1-P receptor, coupled to $G_i/G_o$, or a receptor-independent G protein activation have been proposed (Wu *et al.* 1995).

In the present study, we show that Sph-1-P activates atrial muscarinic $K^+$ channels ($I_{K(ACh)}$) with a high potency ($EC_{50} \approx 1{\cdot}2$ nM). This channel represents the target of a signalling pathway first described in atrial myocytes, consisting of only three components: the muscarinic ($M_2$) receptor, a PTX-sensitive heterotrimeric G protein and the ion channel, the opening activity of which is directly controlled by interaction with the $\beta\gamma$-subunits of the G protein (for reviews, see Kurachi, 1994; Clapham, 1994). $I_{K(ACh)}$ activation by Sph-1-P exhibits characteristics which suggest that it is initiated by binding of the phospholipid to a membrane receptor. As well as reacting with Sph-1-P, the postulated receptor also seems to interact with an albumin-associated $I_{K(ACh)}$-activating phospholipid (Bünemann & Pott, 1993) the nature of which has not been identified yet, but which is apparently different from Sph-1-P. Our results

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demonstrate for the first time Sph-1-P-induced signal transduction via a G protein-coupled membrane receptor in a terminally differentiated cell.

## METHODS

### Isolation and culture of atrial myocytes

Guinea-pigs of either sex weighing 250–300 g were killed by cervical dislocation. The method of enzymatic isolation of atrial myocytes has been described in detail previously (e.g. Banach, Hüser, Lipp, Wellner & Pott, 1993*b*). The culture medium was serum-free bicarbonate-buffered M199 (Gibco) containing gentamicin and kanamycin (Sigma). The cells were plated at a density of several hundred cells per square centimetre on 36 mm culture dishes. Cells were cultured and used experimentally for up to 8 days. Sensitivity to Sph-1-P was not affected by time in culture.

### Solutions

For the measurements, the culture medium was replaced by a solution containing (mM): NaCl, 120; KCl, 20; $CaCl_2$, 2·0; $MgCl_2$, 1·0; Hepes, 10·0, pH adjusted to 7·4 with NaOH. The solution for filling the patch clamp pipettes for whole-cell voltage clamp experiments contained (mM): potassium aspartate, 100; NaCl, 10; KCl, 40; MgATP, 5·0; EGTA, 2·0; GTP, 0·01; Hepes, 10·0, pH adjusted to 7·4 with KOH. Sphingosine and Sph-1-P were from Biomol (Hamburg, Germany).

### Current measurement

Membrane currents were measured under voltage clamp by means of patch clamp pipettes (whole-cell mode; Hamill, Marty, Neher, Sakmann & Sigworth, 1981). Because of its strong inwardly rectifying properties, $I_{K(ACh)}$ was measured as inward current ($E_K = -50$ mV; holding potential $= -90$ mV). Pipettes were fabricated from borosilicate glass with filament (Clark Electromedical, Pangbourne, UK) on a vertical puller (DMZ, Munich, Germany). The DC resistance of the filled pipettes ranged from 2 to 6 MΩ. Currents were measured by means of a patch clamp amplifier (List LM/EPC 7). Signals were analog filtered (corner frequency, 1 kHz) and were digitally stored on the hard disk of a computer, equipped with a hardware/software package (ISO2 by MFK, Frankfurt on Main, Germany) for voltage control, data acquisition and data evaluation. Experiments were performed at ambient temperature (22–24 °C). Rapid superfusion of the cells was performed by means of a solenoid-operated flow system that permitted switching between up to six different solutions. Whenever possible, results are presented as mean values ± standard deviation (S.D.).

## RESULTS

The responses to ACh (10 μM) and Sph-1-P (0·1–100 nM) recorded from a single atrial myocyte are illustrated in Fig. 1*A*. Inward $I_{K(ACh)}$ evoked by a saturating concentration of the vagal transmitter was characterized by a rapidly desensitizing component of variable amplitude in different cells, the mechanism of which is still controversial. The second component, which, at least over a period of time of several minutes, did not further desensitize, will be referred to hereafter as steady-state $I_{K(ACh)}$. The latter saturated at concentrations of ACh >1 μM. Due to its slower onset of action, the desensitizing component was not seen with Sph-1-P. Amplitudes of current evoked by Sph-1-P were normalized to steady-state $I_{K(ACh)}$ in the presence of 10 μM ACh. The total steady-state current densities that were available to either ACh or Sph-1-P were nearly identical in this cell. As shown in Fig. 1*B*, the current–voltage relations determined by voltage ramps from −120 to +60 mV were identical for the currents activated by either ACh or Sph-1-P. They were characterized by the strong inward-going rectification characteristic of this current (Sakmann, Noma & Trautwein, 1983). In cat atrial myocytes under certain conditions a component of membrane current activated by ACh is sensitive to glibenclamide and is therefore likely to be carried by ATP-sensitive ($I_{K(ATP)}$) $K^+$ channels (Wang & Lipsius, 1995). Under the present experimental conditions the currents activated by either ACh or Sph-1-P were completely insensitive to glibenclamide (10 μM). Contamination of $I_{K(ACh)}$ by $I_{K(ATP)}$ can therefore be safely excluded.

The dependence of $I_{K(ACh)}$ activation on the concentration of Sph-1-P is summarized in Fig. 1*C*. The maximum response in this series of measurements was 88·0 ± 7% of that elicited by a saturating concentration of ACh. The response to Sph-1-P was insensitive to the muscarinic antagonist atropine (10 μM), which completely inhibited the response to saturating concentrations of ACh and to the adenosine $A_1$ antagonist theophylline (1 mM, data not shown). These observations exclude a contribution by muscarinic and $A_1$ receptors to the action of Sph-1-P.

Previous studies have shown that the signalling actions of Sph-1-P in fibroblast and smooth muscle cells can be mimicked by sphingosine, which is converted to active Sph-1-P by a kinase presumably located in the ER membrane (e.g. Ghosh *et al.* 1994). Figure 2 illustrates that sphingosine fails to activate $I_{K(ACh)}$ in atrial myocytes. The representative current trace shows inward $I_{K(ACh)}$ caused by ACh (10 μM) and Sph-1-P (100 nM). Sphingosine at a concentration of 1 μM did not result in a measurable inward change in the holding current. This was confirmed in six different cells. A challenge with 10 μM ACh while $I_{K(ACh)}$ was still activated by Sph-1-P resulted in activation of a small additional current that just represented the difference between the maximal $I_{K(ACh)}$ levels that could be evoked by ACh and Sph-1-P. This representative observation confirms that the same population of $I_{K(ACh)}$ channels is recruited by ACh and Sph-1-P.

Activation of $I_{K(ACh)}$ channels by ACh (via muscarinic $M_2$ receptors) and adenosine (via $A_1$ receptors) proceeds in a membrane-delimited fashion by activation of PTX-sensitive G protein, most probably by interaction of the $\beta\gamma$-subunits with a subunit of the channel (Kurachi, 1994; Clapham, 1994). Studies performed on Swiss 3T3 fibroblasts demonstrated that mitogenic signalling, as well as an increase in cytoplasmic $Ca^{2+}$ and a decrease in forskolin-stimulated cAMP accumulation by Sph-1-P, are attenuated by PTX treatment, whereas Sph-1-P-induced

formation of PA was not affected by the toxin (Goodemote *et al.* 1995; Wu *et al.* 1995). In five cells tested, incubation with PTX (2 h at 37 °C; 2 $\mu$g ml$^{-1}$) resulted in complete suppression of the response to both ACh and Sph-1-P. Under control conditions, cells that did not respond to ACh or Sph-1-P were never found.

A common property shared by G protein-linked receptor-mediated signalling pathways is their desensitization upon exposure of a receptor to its agonist (see Premont, Inglese & Lefkowitz, 1995, for review). Usually this desensitization is homologous at the receptor level. As heterotrimeric G proteins and channels involved in the activation of macroscopic $I_{K(ACh)}$ are apparently identical for ACh and Sph-1-P, reduced sensitivity to one agonist only upon long-term exposure to one of the two agonists implies the participation of two distinct $G_i$-coupled receptors. Figure 3*A* illustrates that exposure of the myocytes to the stable muscarinic agonist carbachol (CCh, 10 $\mu$M for 20 h) resulted in an almost complete loss of sensitivity to the standard concentration of ACh (10 $\mu$M). In contrast, the $I_{K(ACh)}$ density assessed by challenging the cells with 100 nM Sph-1-P was not reduced. If cells were pre-incubated with Sph-1-P (100 nM), sensitivity to the phospholipid (100 nM) was significantly reduced without affecting the muscarinic response (Fig. 3*B*). In both cases desensitization reversed only slowly. Within 2 h of removal of the down-regulating treatment no recovery was observed. This slow reversibility distinguishes the desensitization described here from the rapid early decay of $I_{K(ACh)}$ (compare with Fig. 1). Recovery from this 'acute densensitization', which most probably is not a receptor phenomenon, occurs within seconds to a few minutes of wash-out of the desensitizing agonist (e.g. Kurachi, Nakajima & Sugimoto, 1987).

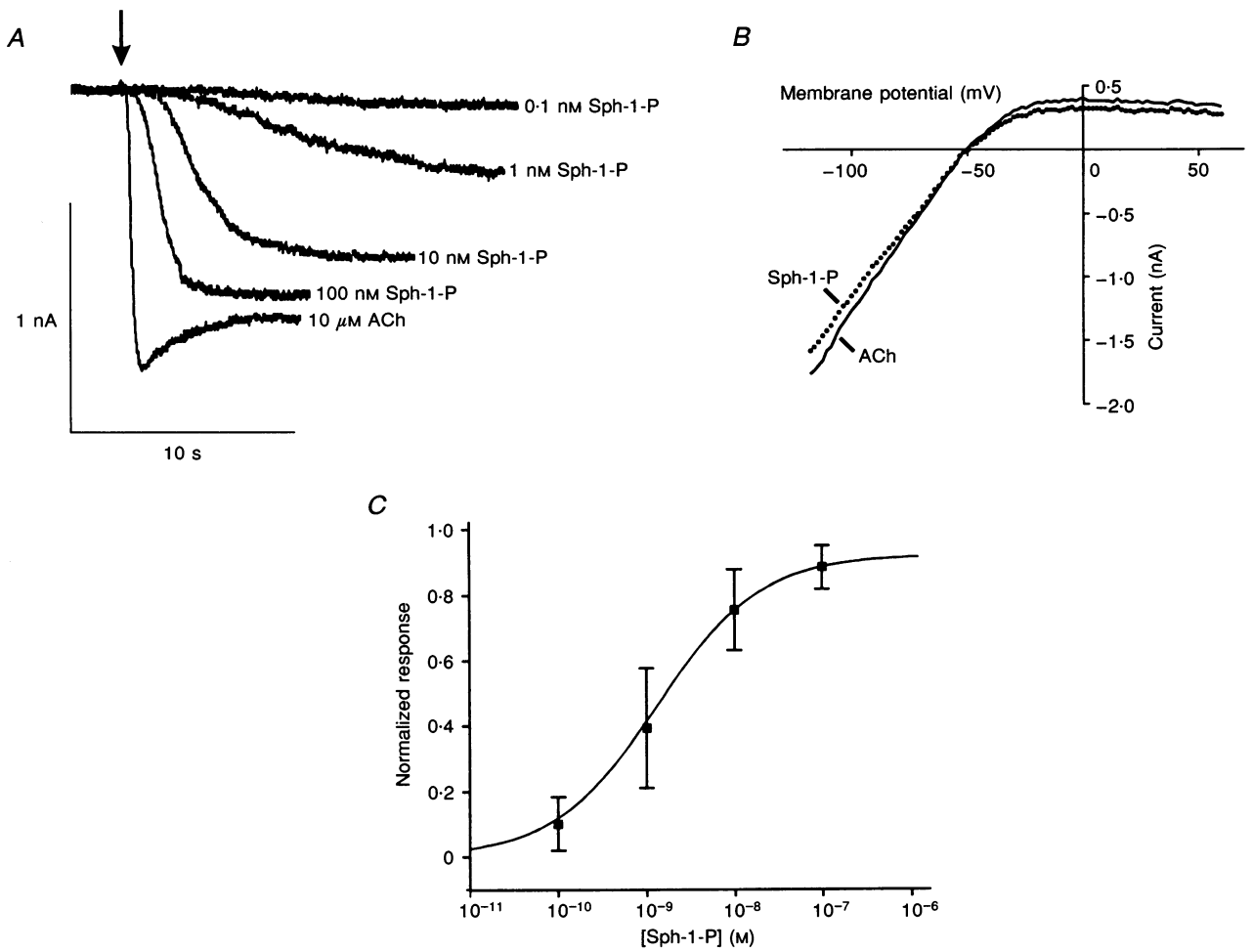


**Figure 1. Activation of $I_{K(ACh)}$ by Sph-1-P**

*A*, onset of inward $I_{K(ACh)}$ upon switching superfusion of a myocyte to solutions containing Sph-1-P at the concentrations indicated, and to 10 $\mu$M ACh. *B*, current–voltage relations of Sph-1-P (100 nM)- and ACh (10 $\mu$M)-activated steady-state currents. Voltage ramps from −120 to + 60 mV (360 mV s$^{-1}$) were applied 20 s after beginning of superfusion with agonist-containing solutions; background *I–V* curves have been subtracted. *C*, concentration dependence of $I_{K(ACh)}$ activation by Sph-1-P. Current amplitudes were normalized to steady-state current in the presence of 2 $\mu$M ACh; $n = 5$.

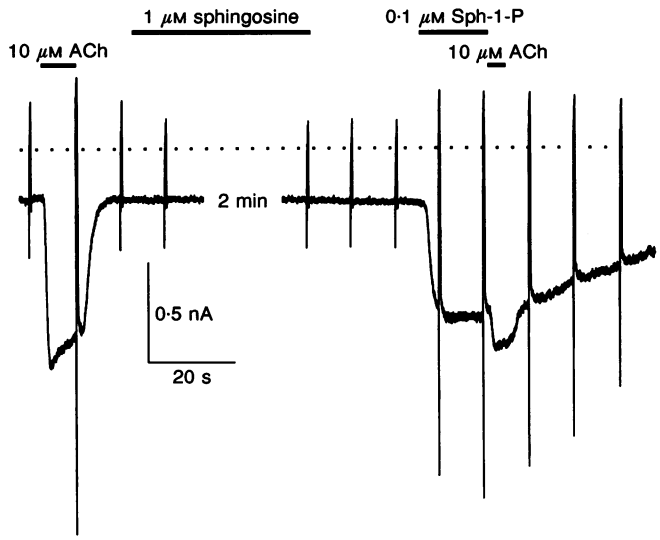


**Figure 2. Sphingosine does not activate $I_{K(ACh)}$**
ACh, sphingosine and Sph-1-P were superfused as indicated. Rapid deflections represent currents due to standard voltage ramps. The dotted line indicates zero current level.

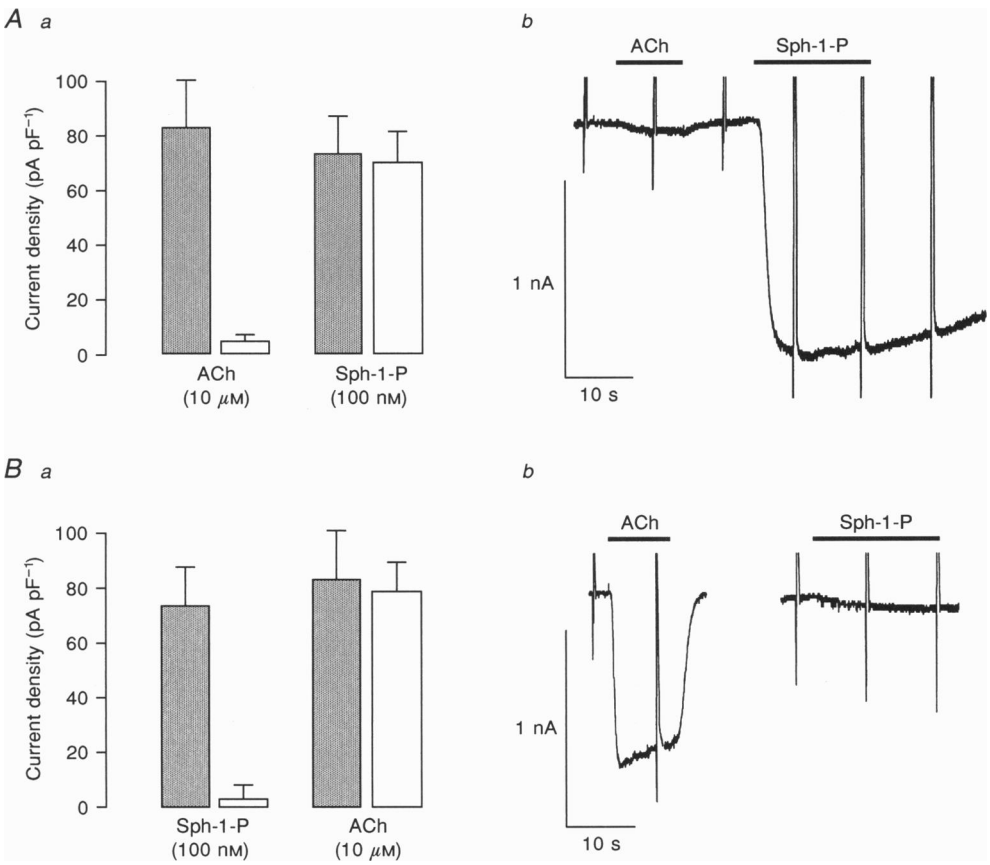


**Figure 3. Homologuous desensitization by ACh and Sph-1-P**
Myocytes were incubated with either CCh (10 μM, 20 h; *A*) or Sph-1-P (100 nM, 2 h; *B*). Thereafter cells were washed with an excess of solution. Measurements were performed within the subsequent 2 h. Desensitized responses did not measurably recover during this period of time. *Aa* and *Ba*, summarized data from ACh-treated and Sph-1-P-treated cells, respectively. ▩, control cells ($n = 6$); □, desensitized cells ($n = 6$ in $A$; $n = 5$ in $B$). *Ab* and *Bb* are representative current traces from either condition.

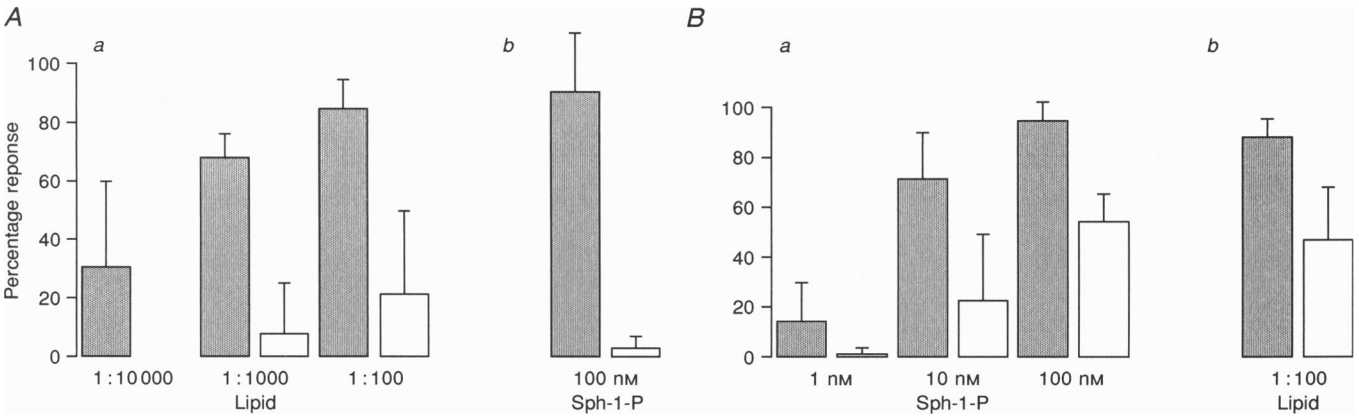


**Figure 4. Cross-desensitization of responses to Sph-1-P and serum phospholipids**

Cells were incubated with culture medium containing either Sph-1-P (100 nM) or a phospholipid mixture purchased from Sigma (L4646; 1:100, which is a concentration that maximally activates $I_{K(ACh)}$). After 2 × 45 min treatment, the cells were washed with an excess of solution. *A*, responses to three different concentrations of lipid mixture in Sph-1-P-treated cells (*a*) and to Sph-1-P (*b*). ▩, control cells ($n = 5$ in *A*; $n = 6$ in *B*); □, desensitized cells ($n = 5$ in *A*; $n = 7$ in *B*). Current amplitudes were normalized to ACh-evoked (10 $\mu$M) current, the density of which was not affected. *B*, analogous data from cells treated with serum phospholipids.

In the case of CCh-treated cells, recovery occurred with a half-time of around 30 h. This homologous desensitization provides evidence that Sph-1-P in the signalling system under study acts via a distinct $G_i$-coupled membrane receptor.

We have previously described an albumin-associated phospholipid which evokes the $G_i$-controlled responses commonly described for mammalian atrial myocytes, namely activation of $I_{K(ACh)}$ channels and inhibition of $\beta$-adrenoceptor-stimulated adenylyl cyclase (Banach, Bünemann, Hüser & Pott, 1993*a*; Banach *et al.* 1993*b*; Bünemann & Pott, 1993). From these results, the existence in atrial myocytes of a phospholipid receptor coupled to a PTX-sensitive G protein has been postulated. The active compound so far has not been identified. A number of phospholipids with signalling properties in other systems such as lysophosphatidic acid, lysophosphatidylcholine and platelet-activating factor have been found to be unable to activate $I_{K(ACh)}$ in atrial myocytes (Bünemann, Ferrebee, Tigyi & Pott, 1994). The data presented above seem to indicate that Sph-1-P represents the hitherto unidentified albumin-associated phospholipid. This, however, can be excluded for the following reason: using a HPLC method for separation of phospholipids, the retention times of the active fraction of serum-derived phospholipids and of Sph-1-P are distinctly different without any overlap of the elution profiles (M. Bünemann, K. Liliom, G. Tigyi & L. Pott, in preparation). Although the serum-derived $G_i$-activating phospholipid is not identical with Sph-1-P, it is conceivable that these two compounds share a common receptor. This question was addressed by a cross-desensitization protocol analogous to the experiments illustrated in Fig. 3. The summarized data from cells that were incubated with Sph-1-P for 90 min and thereafter tested for sensitivity to the standard mixture of serum phospholipids or vice versa are illustrated in Fig. 4. Both treatments resulted in a significant reduction of sensitivity to the desensitizing compound itself, as well as the phospholipid the cells had not been exposed to. Thus, desensitization is 'heterologous' between these two substances. These results suggest that Sph-1-P and the unidentified serum phospholipid act via a common phospholipid receptor.

## DISCUSSION

Measurement of $I_{K(ACh)}$ provides a very sensitive on-line assay for measuring activation of the $G_i$-type G protein in atrial myocytes. The data of the present study provide evidence that atrial myocytes possess a membrane receptor with high affinity for the phospholipid Sph-1-P, coupled to a PTX-sensitive G protein.

A number of different cellular signals related to sphingosine and Sph-1-P have been described previously (see Introduction). Some of the effects of Sph-1-P can be mimicked by sphingosine, and it is proposed that exogenously applied sphingosine enters the cells where it is converted by a sphingosine kinase to Sph-1-P. This represents an important difference between previous findings and those of the present study. Sphingosine at a concentration up to 1 $\mu$M, i.e. $10^3$ times the $EC_{50}$ of Sph-1-P, failed to cause any measurable activation of $I_{K(ACh)}$. Although one cannot conclude from this finding that conversion of sphingosine to Sph-1-P does not occur in atrial myocytes, such a pathway seems to be of no relevance to activation of the G protein that controls gating

of $I_{K(ACh)}$ channels. The second difference between the present study and those quoted above is our finding that PTX treatment completely abolished responses to Sph-1-P. In Swiss 3T3 cells it was found that activation of phospholipase D by Sph-1-P was resistant to PTX; other signals, such as inhibition of cAMP accumulation, stimulation of MAP kinase and increase in cytosolic $Ca^{2+}$ concentration, were reduced but not completely inhibited (Goodemote *et al.* 1995; Wu *et al.* 1995). A third major difference between our data and previous publications on Sph-1-P signalling is the range of effective concentrations of the phospholipid. The $EC_{50}$ for activation of $I_{K(ACh)}$ (1·2 nM) is about three orders of magnitude lower than previously published effective concentrations in the micromolar range. A similarly high potency has been found very recently for PTX-sensitive increases in the cytosolic $Ca^{2+}$ concentration in human embryonic kidney cells (van Koppen *et al.* 1995). Although the partial sensitivity of Sph-1-P-induced effects to PTX has suggested the existence of a G protein-coupled membrane receptor for Sph-1-P (Goodemote *et al.* 1995), other alternative pathways have been discussed by these authors, such as a receptor-independent G-protein activation via Sph-1-P-induced perturbation of the lipid bilayer.

Homologous desensitization at the level of the membrane receptor is a common finding in systems where different receptors converge on one type of G protein. It is primarily caused by a receptor kinase which recognizes only agonist-occupied receptors as substrate molecules for phosphorylation (see Premont *et al.* 1995 for review). In atrial myocytes, using $I_{K(ACh)}$ as an indicator, $M_2$ receptors can be completely desensitized leaving the response to the $A_1$ agonist adenosine unaffected and vice versa (Bünemann & Pott, 1995; M. Bünemann & L. Pott, in preparation), suggesting that desensitization within the short signalling pathway under consideration occurs only at the level of the receptor(s). In the present study pre-incubation of the myocytes with Sph-1-P caused desensitization of a response to an acute challenge with the phospholipid. This desensitization was homologous, i.e. sensitivity to the muscarinic agonist ACh was not affected. On the other hand, the albumin-associated phospholipid previously described as possessing muscarinic activity in atrial myocytes, which apparently is not identical with Sph-1-P, cross-desensitized with Sph-1-P. These findings further support the view of the existence of a membrane receptor as primary target of Sph-1-P and the unidentified albumin-associated phospholipid in atrial myocytes.

The major role of $I_{K(ACh)}$ is to hyperpolarize supraventricular tissue, which contributes to regulating the frequency of action potential discharge in pacemaker cells. The physiological role of activation of this system by Sph-1-P or other active phospholipids (Bünemann & Pott, 1993) is completely unknown at present. From the finding that the effects of Sph-1-P can be seen at concentrations in the subnanomolar range, a mechanism with a high degree of specificity seems to be obvious. However, at present we do not even know whether Sph-1-P is the physiological ligand at the postulated receptor. It is conceivable that it mimics a structurally related compound. Whatever the natural ligand is, further investigations as to its identity and its source are required in order to understand the physiological role of the phospholipid receptor. Previous results on Sph-1-P-induced cellular signals, primarily in fibroblast cell lines, have suggested a role for this phospholipid in the control of cell proliferation. The question, however, of when and how these signals are used to regulate the function or state of a cell in the organism for those systems has not so far been answered.


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