

taneously at the tails and the origin) for the lognormal with the above parameters.

In the figure we graph the expected time to the next incident as a function of operating experience; the lognormal prior uses the left-hand scale. For simplicity we assume that the first and second incidents occur at 3,000 and 4,000 reactor years, respectively, and that one calendar year with today's inventory of operating nuclear reactors corresponds to about 400 reactor years as of 1985. At the second incident the conditional expectation decreases to about 3,000 reactor years. Not only is there a decrease following each incident but it should be noted that the slope of each successive line segment decreases over time. This is due, very simply, to the fact that the non-occurrence of an incident in late time periods carries less information than non-occurrence of incidents did in the early lives of nuclear reactor operations. The graph that corresponds to the uniform prior used in the earlier articles by Islam and Lindgren and Edwards makes use of the right-hand scale.

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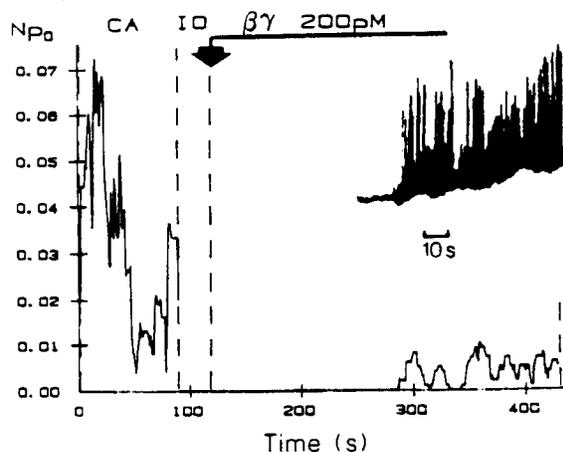
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## G protein opening of K<sup>+</sup> channels

STR—Logothetis *et al.*<sup>1</sup> claim that  $\beta\gamma$ -subunits of G proteins acting at nanomolar concentrations are the mediators of the activation of K<sup>+</sup> channels by muscarinic acetylcholine receptors (mAChR) in the heart. The conclusion is unsettling, as noted in the News and Views article by Bourne<sup>2</sup>, because it raises a difficult question — how does an agonist specifically activate K<sup>+</sup> channels when it uses free  $\beta\gamma$  that can be released from several G proteins? The solution to the quandary turns out to be simple. It is provided by our discovery<sup>3</sup> that the G protein, G<sub>i</sub>, stimulates mammalian atrial K<sup>+</sup> channels at picomolar concentrations whereas pre-activated G<sub>i</sub>, which has the same  $\beta\gamma$  as G<sub>i</sub> but differs in its  $\alpha$ -subunit, has no effect at concentrations several hundred times greater. Specificity thus comes from the  $\alpha$  confirmed by Hescheler *et al.*,<sup>4</sup> who show that the  $\alpha$  and not the  $\beta\gamma$  of avian brain G<sub>o</sub> regulates neuronal calcium channels<sup>5</sup>.

So why did Logothetis *et al.* find that  $\beta\gamma$  are stimulatory and that  $\alpha$  is without

Cell-attached (CA) and inside-out (IO) patch activity from a 14-day old chick atrial cell. 200 pM  $\beta\gamma$  activates the channel. Channel amplitudes, 2.9 pA.



effect but when mixed with  $\beta\gamma$  quenches its effect? The answer does not lie in the electrophysiological data as the particular K<sup>+</sup> channels involved can be identified unambiguously. It probably lies in the nature of the G protein preparations used to obtain the data and we offer the following explanations.

First, the  $\alpha$  was either the wrong one, partially denatured, or subjected to an inadequate activation protocol. Neer *et al.* originally described three substrates for pertussis toxin (PTX) in brain<sup>6</sup>, but only two were tested by Logothetis *et al.* No data were given as to whether the activation protocol used to convert  $\alpha$ -GDP complexes to  $\alpha$ -GTP $\gamma$ S complexes actually resulted in nucleotide exchange. Apparently, 500 to 1280 nM  $\alpha$  were treated for 30 min at 22 °C with 200 nM guanine nucleotide in the presence of 2 mM Mg<sup>2+</sup>. In our hands this would lead to little if any nucleotide exchange and to some inactivation.

Second, a contaminant, not the approximately 23 nM  $\beta\alpha$ , may have caused opening of K<sup>+</sup> channels. The contaminant could be a phospholipase that, on modifying the lipid environment of the channel, causes it to open, or an  $\alpha\beta\gamma$ -G protein, such as G<sub>o</sub>, that is active in opening K<sup>+</sup> channels. Phospholipases are known to mimic certain hormonal effects at the plasma membrane<sup>4</sup>. Contamination of the  $\alpha$  preparations with a heat labile protease could then account for the quenching of  $\beta\gamma$  were the phospholipase or G<sub>o</sub> sensitive to the protease and the protease insensitive to the inhibitors that were used. Although the exact details may differ, the point is that contaminants could account for the results.

As to the possible presence of G<sub>o</sub>, no attempt to quantify contamination by a PTX-sensitive  $\alpha\beta\gamma$  in the  $\beta\gamma$  preparations is described, other than a Coomassie blue stain of a gel to which 2  $\mu$ g of  $\beta\gamma$  had been applied. This method would not allow detection of < 0.1  $\mu$ g  $\alpha$ ; methods to increase the sensitivity of the assay 100–1,000-fold are available<sup>5</sup>. Although Logothetis *et al.*

state that  $\beta\gamma$  prepared by two different procedures was used, citation to only one is given. In it, extracted G proteins are stabilized with fluoride, aluminium chloride and Mg<sup>2+</sup>. For G<sub>o</sub>, with which this can be measured, this type of treatment leads to a preactive state — undissociated in the cold yet active in *cyc*<sup>-</sup> reconstitution assays without addition of activating ligands<sup>7</sup>. It is therefore possible that  $\alpha\beta\gamma$  complexes contaminating  $\beta\gamma$  at ratios between 1 in 10,000 and 1 in 1,000 would be active on K<sup>+</sup> channels as tested by Logothetis *et al.* We have observed activation of K<sup>+</sup> channels of the type they obtained with 20 nM  $\beta\gamma$  using only 20–100 pM GTP $\gamma$ S-activated G<sub>i</sub>. Furthermore, in our hands 2 nM of a GTP $\gamma$ S-activated preparation of G<sub>o</sub>, which is an equimolar mixture of  $\alpha$ -GTP $\gamma$ S plus the same amount of free  $\beta\gamma$ , failed to elicit any K<sup>+</sup> channel openings. Because the  $\beta\gamma$  complexes in G<sub>i</sub> and those in our PTX-sensitive G protein are the same<sup>7,8</sup> we emphasize that it is the PTX-sensitive  $\alpha$ -GTP $\gamma$ S complex of G<sub>i</sub>, and not the  $\beta\gamma$  complex that is responsible for mediating the effect of G<sub>i</sub>, and hence for the action of muscarinic receptors on K<sup>+</sup> channels.

The data of Logothetis *et al.* raise the question as to whether high concentrations of  $\beta\gamma$  can indeed activate K<sup>+</sup> channels in embryonic chick atrial membranes. If so, it will be necessary to determine whether this is related to the ontogenic state of the cardiac muscarinic response, to the fact that avian instead of mammalian cells were used, or to the need for *in vitro* culture of the cells before they are used.

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**LOGOTHETIS ET AL. REPLY** — Although Birnbaumer and Brown make no such claim in their paper<sup>1</sup>, they now maintain that  $\alpha_w$  is the subunit of  $G_i$  which activates the  $K^+$  channel. They reported no experiments with resolved  $G_i$  subunits, only with  $\alpha\beta\gamma$ -heterotrimers. In contrast, we studied purified  $\alpha$  or  $\beta\gamma$  and found that the  $\beta\gamma$  activates the  $K^+$  channel. We were surprised that  $\beta\gamma$  mediated the  $K^+$  channel activation because we also began with the assumption that  $\alpha$  would be the only effector. Birnbaumer and Brown view our findings and theirs as necessarily mutually exclusive. We disagree and suggest that the truth may be more complicated and more interesting. Our negative results cannot rule out some role for  $\alpha$  subunits, but our positive findings with  $\beta\gamma$  at pM concentrations strongly support a role for  $\beta\gamma$  in activating the  $K^+$  channel in the heart. The challenge for the future will be to define precise functions for the subunits.

Contrary to the assertion of Birnbaumer and Brown, our results with  $\beta\gamma$  are not due to contaminating  $\alpha$  protein. The figure shows that 200 pM  $\beta\gamma$ , a concentration 100-fold lower than we used previously, results in activation equivalent to 200 pM  $G_i$  (Fig. 1, ref. 1). We calculate there is less than 0.5 per cent contamination of  $\alpha$  in the  $\beta\gamma$  preparation, both from densitometry of a highly overloaded SDS-polyacrylamide gel and by pertussis toxin catalysed [<sup>32</sup>P]ADP-ribosylation. If a contaminating  $\alpha$  is responsible, it must be strongly activating the  $K^+$  channel at less than 1 pM, a concentration below that at which Yatani *et al.* see effects of  $G_i$  on the  $K^+$  channel. It is important to stress that any residual  $\alpha$  subunit in our  $\beta\gamma$  preparations would be unactivated. Our  $\beta\gamma$  preparations have never been in GTPyS. Fluoride activation of the  $\alpha_w$  protein is readily reversible by removal of fluoride<sup>2</sup>. Fluoride was removed from the  $\beta\gamma$  preparations by one (or two) steps of gel filtration or chromatography before use. Although our results are now shown to occur in the same concentration range as those cited by Yatani *et al.*, there may well be differences due to different patch channel density, detergent, methods of protein determination or sources of atrial membranes and G proteins.

Birnbaumer and Brown suggest that our inability to see activation of the  $K^+$  channel by pure  $\alpha$  subunits is because they are denatured or unactivated. We tested the function of the particular preparations of  $\alpha_w$  and  $\alpha_{11}$  used. The  $\alpha_w$  subunit was activated at 5mM  $Mg^{2+}$ , not at 2mM  $Mg^{2+}$ . It has a brisk GTPase activity at 22°C which is inhibited by 200 nM GTPyS. Thus, nucleotides are turning

over at the active site. Although the  $\alpha$  subunits were in excess of GTPyS during activation, we calculate from the  $K_D$  (30 nM) that we determined<sup>3</sup> for  $\alpha_w$  and  $\alpha_{11}$  that the solution applied to the patch contained approximately 9 nM GTPyS-liganded  $\alpha$  subunit, a concentration about 40 times greater than necessary to activate  $K^+$  channels according to Yatani *et al.* The minor (40K) pertussis toxin substrate from brain, described by us<sup>4</sup> has not yet been purified but is always present in small amounts in the  $\alpha_w$  preparation.

A strong control for the specificity of the action of  $\beta\gamma$  is inhibition of its effect by  $\alpha$  subunits, presumably by the formation of inactive heterotrimers. Birnbaumer and Brown speculate that a contaminating protease in the  $\alpha$  preparation is responsible. But when we incubate our  $\alpha$  with  $\beta\gamma$  for 40 min at 30 °C we find no breakdown of either subunit on subsequent SDS-PAGE. Birnbaumer and Brown's suggestion that a protease in the  $\alpha$  preparation inactivates a phospholipase in the  $\beta\gamma$  preparation is sheer speculation. Finally, the observation that  $\alpha_w$  decreases Ca channel conductance in NG108 cells<sup>5</sup> has nothing to do with  $K^+$  channels in heart cells.

Thus we have activated the  $K^+$  channel with picomolar concentrations of  $\beta\gamma$  subunits alone. GTPyS-liganded  $\alpha$  subunits at nM concentrations did not activate the channel. Birnbaumer and Brown should test resolved subunits to determine individual activities of their  $\alpha_w$  and  $\beta\gamma$ .

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## Climate and reproduction of yellowstone grizzlies

**SIR**—For more than 50 years before 1968, grizzly bears were permitted free access to several large open-pit garbage dumps inside Yellowstone National Park. Throughout the summer, grizzlies congregated at these sites in large numbers (up to 170 individuals) to feed undisturbed on refuse. Between 1968 and 1971 these dumps were closed abruptly and since then most Yellowstone grizzlies have had to find sufficient natural summer forage to compensate for the loss of garbage. This situation may suit many conserva-

tionists and land managers; but it has almost certainly imposed additional hardship on a population beset with low reproductive rate, high human-induced mortality<sup>1</sup> and severe habitat encroachment.

Picton analysed climate and reproduction of grizzly bears in Yellowstone National Park<sup>2</sup> and concluded that climatic effects, specifically temperature and precipitation from October to May, were responsible for the decline in grizzly bear litter sizes observed since 1972. We contend that the abrupt closure of the open-pit garbage dumps was a more likely cause of this decline.

To evaluate the relative effects of climate and dump closures on grizzly reproduction properly, climatic factors should be controlled for by analysis of covariance. Using such an analysis, Knight and Eberhardt<sup>1</sup> recently showed that after adjusting for differences in climate, mean annual litter sizes were significantly larger from 1959 to 1970 than from 1971 to 1981. This suggests that the dump closures, rather than climate, probably caused the decline. Our own analysis of covariance shows that when individual litter sizes are adjusted to a common climate index, the mean for the years before the dump closures (1959-69,  $x_{adj}$  = 2.17 cubs per litter) was significantly larger than the mean for the years 1973-1981 ( $x_{adj}$  = 1.95,  $P=0.02$ ; manuscript in preparation). We omitted litters born between 1970 and 1972 because they were conceived during the period of major dump closures (1969-71)<sup>3</sup>.

Picton<sup>2</sup> further considered the combined effects of climate and carrion on mean grizzly litter sizes. Because these two predictor variables are undoubtedly inter-correlated, we question the validity of combining the two as he did. It would be more appropriate to control for the effects of each variable statistically, using either path analysis or a partial correlation analysis. We have been unable to do this, however, because neither the carrion data nor the method of deriving an index of relative availability is accessible. The indices themselves are in press<sup>4</sup>, but they include only the values of +1 and -1, suggesting little variation among years in carrion availability. Presumably there are valid data underlying these indices, but Picton has yet to make them available or describe precisely how the indices were derived.

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